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PREDICCIÓN DE LA RESISTENCIA A ANTIBIÓTICOS, INTRÍNSECA Y ADQUIRIDA, EN *PSEUDOMONAS AERUGINOSA*

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Tesis presentada por

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A mi abuelo.

“Sólo podemos elegir qué hacer con el tiempo que se nos ha dado”
-Gandalf-

“Somos nosotros. Sólo nosotros”
-Rorschach-

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RESUMEN

Resumen

La resistencia a antibióticos ha sido históricamente un escollo para el tratamiento de las infecciones bacterianas, mas en décadas recientes se ha visto recrudecida hasta constituir un grave problema de salud pública, debido a la emergencia y diseminación de bacterias multirresistentes, máxime las Gram-negativas. Estos microorganismos pueden resistir intrínsecamente a los antibióticos; y adquirir resistencia vía transferencia genética horizontal o mediante mutaciones. Entre ellos se encuentra *Pseudomonas aeruginosa*: se trata de un patógeno oportunista ampliamente distribuido en la Naturaleza, que infecta con frecuencia a pacientes hospitalizados y que posee, intrínsecamente, una baja sensibilidad a muchos antibióticos, aparte de desarrollar habitualmente resistencia vía mutaciones durante las infecciones crónicas. Ergo, los estudios predictivos de resistencia intrínseca y adquirida a antimicrobianos clínicamente relevantes en este patógeno son de crucial importancia.

El escrutinio de genotecas de inserción es un método útil en el estudio del resistoma intrínseco bacteriano, pero se desconoce si los resultados que proporciona referentes a un antibiótico pueden extrapolarse al resto de su familia estructural. En esta tesis se ha abordado este interrogante en el caso del resistoma intrínseco a aminoglicósidos de *P. aeruginosa*, hallando no aplicable dicha extrapolación. Adicionalmente, un análisis ulterior reveló que un mutante de pérdida de función de *glnD* presentaba hipersensibilidad a dispares antibióticos y virulencia morigerada, lo que posiciona a este gen como una diana prometedora para el diseño de coadyuvantes de antibióticos frente a *P. aeruginosa*.

Por otra parte, a fin de desvelar los mecanismos implicados en la adquisición de resistencia (vía mutación) a distintos antimicrobianos en *P. aeruginosa*, esta bacteria fue sometida a evolución experimental en presencia de tobramicina, tigeciclina, ceftazidima o ceftazidima-avibactam. Las rutas evolutivas adoptadas por las poblaciones en todas las condiciones exhibieron un notable grado de conservación. A la par, se ahondó en algunos de los factores que constriñen la evolución de la resistencia a tobramicina y tigeciclina. Por un lado, se determinó que la presión de selección condiciona las trayectorias evolutivas hacia dicha resistencia en este patógeno. Aparte, se profundizó en el rol que posee la epistasia entre determinantes de virulencia y de resistencia a los antibióticos en delimitar la evolución de esta última. Los resultados apuntaron a la existencia de contingencia recíproca entre ambos atributos.

Al respecto de las evoluciones realizadas en presencia de β-lactámicos, éstos seleccionaron prevalentemente mutaciones que dieron lugar a la sobre-expresión de *mexAB-oprM*, y grandes deleciones cromosómicas. Estas últimas estaban aparejadas a un *trade-off* evolutivo (sensibilidad colateral) robusto, que podría explotarse en el tratamiento de ciertas infecciones causadas por *P. aeruginosa*, incluso las que contienen mutantes resistentes a diferentes drogas.

En suma, los resultados que esta tesis comprende arrojan luz sobre algunos de los mecanismos de resistencia intrínseca y adquirida por mutaciones en *P. aeruginosa*, así como ciertos factores restrictivos de su evolución, cuyo análisis podría mejorar su predicción.

Summary

Antibiotic resistance (AR) has historically been a hurdle for the treatment of bacterial infections, but in recent decades the situation has been aggravated till becoming a major public health concern, because of the emergence and spread of multidrug resistant bacteria, especially Gram-negative ones. These microorganisms can be intrinsically resistant to antibiotics; and they can acquire resistance by horizontal gene transfer or by mutations. Among them, *Pseudomonas aeruginosa* stands out: it is an opportunistic pathogen widely distributed in Nature, which frequently infects hospitalized patients and exhibits intrinsically low susceptibility to many drugs; moreover, it usually develops resistance via mutation when causing chronic infections. Hence, the prediction studies of *P. aeruginosa* intrinsic and acquired resistance to clinically relevant antimicrobials are of crucial importance.

High-throughput screening of transposon insertion libraries is a useful method to study the bacterial intrinsic resistome, but it is not well known if the results it provides for one antibiotic can be extrapolated to the rest of its structural family. In this thesis, this issue has been addressed in the case of the *P. aeruginosa* intrinsic resistome to aminoglycosides, finding said extrapolation to be inapplicable. In addition, further analysis revealed that a loss-of-function mutant in *glnD* presented hypersusceptibility to different antibiotics and a strongly impaired virulence, which positioned this gene as a promising target for the design of antibiotic coadjuvants against *P. aeruginosa*.

Conversely, in order to unveil the mechanisms involved in the acquisition of mutation-driven AR to disparate drugs in this bacterium, *P. aeruginosa* was submitted to experimental evolution in the presence of tobramycin, tigecycline, ceftazidime and ceftazidime-avibactam. The evolutionary routes followed by the populations in all conditions showed a notable degree of conservation. Besides, some of the factors that constrain evolution to tobramycin and tigecycline resistance were delved into. On the one hand, it was observed that selection strength influences the patterns of such evolution toward resistance in this pathogen. Further, the role that epistasis between virulence and AR determinants possesses in restricting evolution of the latter was deciphered. The results revealed the existence of reciprocal contingency between both traits.

Regarding the evolutions carried out in the presence of β-lactams, these drugs selected prevalently for mutations leading to *mexAB-oprM* overexpression, as well as large chromosomal deletions. These deletions were linked to a robust evolutionary trade-off (collateral sensitivity), which may be exploited in the treatment of certain *P. aeruginosa* infections, even the ones that contain resistant mutants to different drugs.

Overall, the results encompassed in this thesis shed light on some of the mechanisms of intrinsic and acquired mutation-driven resistance to antibiotics in *P. aeruginosa*, as well as on certain evolutionary constraints toward AR, which study may ameliorate its prediction.

Abreviaturas

ADN: ácido desoxirribonucleico

ALE: del inglés *Adaptive Laboratory Evolution*

ARN: ácido ribonucleico

CMI: concentración mínima inhibitoria

CMS: concentración mínima de selección

CPM: concentración preventiva de mutantes

EPOC: enfermedad pulmonar obstructiva crónica

ESKAPE: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* y *Enterobacter* spp.

FDA: del inglés *Food and Drug Administration*

FQ: fibrosis quística

HGT: del inglés *Horizontal Gene Transfer*

Kb: kilobase

LPS: lipopolisacárido

MDR: del inglés *MultiDrug Resistance*

MGE: del inglés *Mobile Genetic Element*

OMS: Organización Mundial de la Salud

PBP: del inglés *Penicillin Binding Protein*

QS: del inglés *Quorum Sensing*

RND: del inglés *Resistance Nodulation Division*

ROS: del inglés *Reactive Oxygen Species*

SIDA: síndrome de inmunodeficiencia adquirida

TOTEM: del inglés *Top TEn resistant Microorganisms*

UCI: Unidad de Cuidados Intensivos

Índice

INTRODUCCIÓN

1. Introducción

1.1. Resistencia a los antibióticos: un panorama pesadillesco

Los antibióticos son, sin sombra de duda, uno de los descubrimientos más coruscantes en la historia reciente de la medicina. Su aplicación en clínica para el tratamiento de enfermedades infecciosas ha contribuido a salvar innúmeras vidas desde la introducción de la penicilina en los años 40 [1]. Sin embargo, antes incluso del uso extensivo de estas drogas, ya existían evidencias que apuntaban a la posibilidad de su degradación enzimática por parte de ciertas bacterias [2]. Esto fue sólo el exordio del acuciante problema de salud global que constituye hoy en día la resistencia a los antibióticos [3].

La resistencia a los antibióticos tiene lugar cuando un antimicrobiano fracasa en su función de eliminar un determinado microorganismo o inhibir su crecimiento [3]. Es reconocida como uno de los mayores retos para la salud global del siglo XXI por los organismos de regulación económica y política más insignes, como el Fondo Monetario Internacional, la Organización Mundial de la Salud (OMS), el Banco Mundial o el G8. Todos ellos concuerdan en que se trata de un problema que trasciende el entorno clínico, dado que el ser humano, los animales y el medio ambiente son hábitats interconectados que pueden espolear la emergencia, evolución y propagación de las bacterias resistentes y genes de resistencia, lo que globaliza la situación [4,5]. Por ende, recientemente se han propuesto dos conceptos complementarios, *One Health* y *Global Health* [6], como los nuevos prismas desde los que analizar y, por tanto, combatir la resistencia a antibióticos. Ambos abordajes radican en la idea de la interdependencia entre la salud animal, humana y de los ecosistemas en los que cohabitamos y/o vertemos nuestros residuos, pero, como indica la Figura 1, difieren en su campo de aplicación. La perspectiva *One Health* se centra en la implementación de intervenciones locales, dentro de ecosistemas conectados geográficamente, mientras que la concepción *Global Health* recomendaría políticas ideadas para ser integradas a nivel internacional.

Una de las causas principales adjudicadas para explicar la resistencia a los antibióticos es el exacerbado abuso en su consumo durante las últimas décadas, mas no es la única [7]. El uso inapropiado de antimicrobianos en concentraciones subterapéuticas, que pueden no sólo ser ineficaces, sino promover la selección de resistencia [8]; la aplicación de estos fármacos en el ámbito ganadero y agrícola [5], en ocasiones con propósitos no terapéuticos; las diferentes legislaciones, extraordinariamente laxas en algunos países; o la paulatina disminución en la inversión destinada a acometer el desarrollo de nuevos antibióticos, son algunos de los motivos que esclarecen por qué se ha llegado a la situación de hogaño [9]. Asimismo, el incremento de la población, la globalización y la falta en muchos lugares de sistemas eficaces de tratamiento de residuos humanos o animales, que eventualmente contienen bacterias resistentes o genes de resistencia, pueden fomentar la diseminación de dicha resistencia [10]. Ahora bien, debe puntualizarse que, aunque estas circunstancias están indudablemente acelerando su emergencia y

propagación, la resistencia a los antibióticos es una consecuencia directa del uso de los mismos y de la capacidad de adaptación de las poblaciones bacterianas. Aun en la idílica situación de un manejo óptimo de los antibióticos, las bacterias desarrollarían resistencia a éstos. Tal problema se ha paliado históricamente apelando a la estrategia de la Reina Roja [11]: correr lo más célere posible (desarrollar nuevos antibióticos) para permanecer en el mismo lugar (contrarrestar la resistencia) [12]. Así pues, en la presente situación de carestía de nuevos antimicrobianos, se hace necesario el uso de nuevas estratagemas en la lid contra la resistencia a los antibióticos.

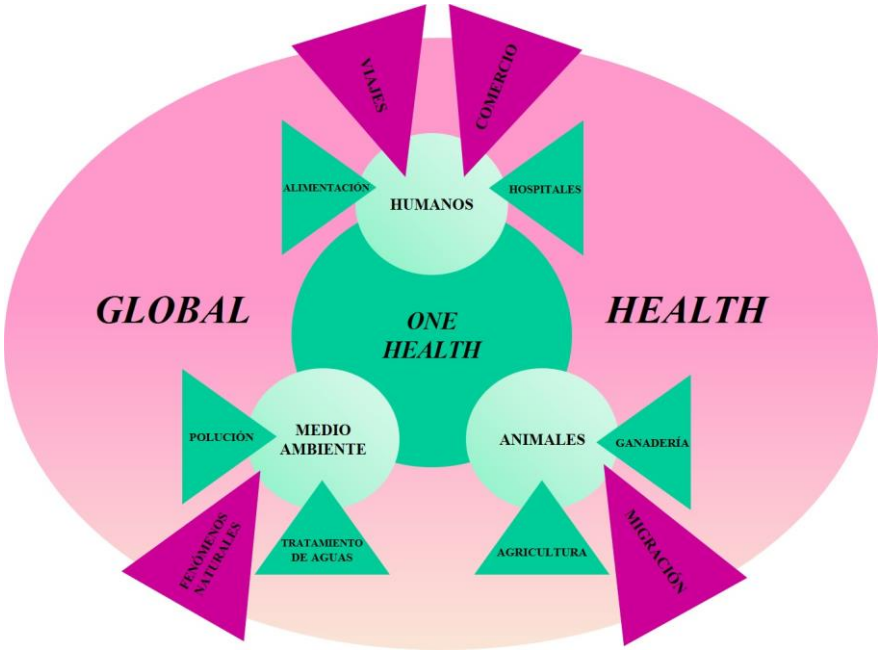

Figura 1. Esquema de las perspectivas *One Health* y *Global Health*, desde las que se propone atajar el problema de la resistencia a antibióticos. La perspectiva *One Health* enfatiza la interdependencia de la salud de los ecosistemas que conforman un ámbito geográfico, y sus medidas se dan a través de la promulgación de leyes a escala local. Por su parte, la perspectiva *Global Health* remarca la comunicación entre dichos ecosistemas locales, los distintos entornos *One Health*, y las condiciones que pueden promover la diseminación de la resistencia a escala mundial. En su caso, las medidas a adoptar son esencialmente recomendaciones, a razón de las disímiles regulaciones de cada país y la falta de sistemas globales de gobernanza.

Como remate, la tesitura todavía se oscurece más ante la existencia de microorganismos que han desarrollado la capacidad de resistir múltiples antibióticos, adquiriendo un fenotipo multirresistente (*MultiDrug Resistance*: MDR). Concretamente, se estima que el número de decesos ocasionados por patógenos MDR orbita en torno a 33000 y 35000 al año en Europa y EE. UU., respectivamente [13,14]. Por consiguiente, esta amenaza ha pasado a recibir la vitola de prioritaria por parte de la OMS [15], confeccionándose compilaciones de bacterias cuya incidencia en la resistencia a antibióticos es particularmente alarmante y contra las que se han de focalizar esfuerzos para hallar nuevos fármacos que las erradiquen exitosamente. Es el caso de las englobadas en los acrónimos ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* y *Enterobacter* spp.) [16] o TOTEM (*TOp TEn resistant Microorganisms*), este último adscrito a las UCIs [17].

1.2. Tipos de resistencia a antibióticos

Los mecanismos de resistencia a antibióticos pueden encasillarse en tres categorías: resistencia intrínseca, resistencia adquirida y resistencia fenotípica o adaptativa [18], si bien un determinante de resistencia puede pertenecer a más de una clase. Seguidamente, se procederá a desglosar cada categoría.

1.2.1. Resistencia intrínseca

Basándose en la definición clínica de la resistencia a los antibióticos, que viene marcada por un umbral establecido de concentración mínima inhibitoria (CMI), un microorganismo es considerado intrínsecamente resistente a una droga cuando ésta es ineficaz frente a la infección que genera. No obstante, si se opta por la más reciente óptica ecológica sobre el término, que instaura el valor umbral de CMI en aquel que presenta la cepa parental de una especie bacteriana [19], el resistoma intrínseco pasa a ser definido como la antología de elementos cromosómicos que contribuyen directa o indirectamente a la resistencia nativa a antibióticos de una bacteria, perteneciendo inherentemente a ésta y no habiendo sido adquiridos por transferencia genética horizontal (*Horizontal Gene Transfer*: HGT) ni modificados por mutación [20,21]. Resulta interesante que bacterias intrínsecamente resistentes a varios antibióticos, como *Stenotrophomonas maltophilia* o *P. aeruginosa*, cuenten con un origen ambiental. Este hecho sugiere que la función fisiológica ancestral de los elementos que constituyen el así llamado resistoma intrínseco no era dotar a las bacterias de resistencia frente a los antibióticos presentes hoy en clínica, sino que éste es un nuevo desempeño desarrollado a resultas de su utilización [20,22].

El resistoma intrínseco abarca habitualmente un maremágnum de genes [21,23-25], que aproximadamente cubren el 3% del genoma bacteriano [26]. Sin embargo, existen tres mecanismos de resistencia que descollan entre esta variedad, dada su recurrencia: ausencia de diana del antibiótico o presencia de un alelo resistente codificante de ésta, enzimas inactivantes del antibiótico no adquiridas por HGT, y merma en la concentración intracelular del fármaco, a causa de una permeabilidad de membrana reducida (especialmente en bacterias Gram-negativas) o de la actividad de bombas de expulsión múltiple de drogas (Figura 2) [18,27].

En lo que al primer caso atañe, el ejemplo más representativo vendría dado por el lipopéptido daptomicina, que es activo frente a bacterias Gram-positivas pero no así frente a Gram-negativas. La explicación a esta particularidad reside en la composición de la membrana, diana de este fármaco: las bacterias Gram-negativas alojan una menor proporción de fosfolípidos aniónicos en su membrana, los cuales son indispensables para la inserción y subsiguiente acción antibacteriana de la daptomicina [28]. Por otro lado, puede darse la situación en que la diana esté presente, pero sea insensible al efecto del antibiótico. El biocida triclosán ilustra perfectamente esta posibilidad: su bajo efecto frente al género *Pseudomonas* (pese a ser activo contra muchas otras bacterias Gram-

negativas), nace de la existencia de un alelo resistente de la enzima enoil-ACP reductasa, diana del triclosán, en este género bacteriano [29].

Otro mecanismo de resistencia intrínseca a antibióticos consiste en la acción de enzimas inactivantes de éstos. Estas enzimas suelen hallarse codificadas en elementos genéticos móviles (*Mobile Genetic Elements*: MGEs), pudiendo ser entonces adquiridas mediante HGT; lo que no obsta para que también puedan localizarse en el cromosoma, sin signo de adquisición reciente, ergo formando parte del resistoma intrínseco. Según su modo de acción, se clasifican en hidrolasas, transferasas y oxidorreductasas [30]. Entre las hidrolasas sobresalen las β-lactamasas -subdivididas en cuatro clases: A, B, C, y D; a razón de su secuencia de aminoácidos [31]- y las macrólido esterasas, que escinden hidrolíticamente el anillo β-lactámico de los antibióticos del mismo nombre o el anillo de lactona de los macrólidos, respectivamente [30,32]. Un ejemplo muy sonado de β-lactamasa, con una alta representación en la familia de las *Enterobacteriaceae* -pese a que éstas crecen en ecosistemas exentos de productores conocidos de β-lactámicos-, es AmpC [33]. Por su parte, las transferasas cuentan como máximas valedoras a las enzimas modificadoras de aminoglicósidos, así como de cloranfenicol, rifampicinas, fosfomicina o macrólidos, que ejercen su efecto mediante la adición de un radical químico que modifica la molécula, tornando por tanto fútil su acción [34]. Finalmente, la oxidorreductasa más destacable es TetX, que dota de resistencia frente a todas las tetraciclinas, al promover la ciclación y posterior descomposición de estas moléculas [34].

Como postrera opción, la resistencia intrínseca puede deberse a una mengua en la concentración de antibiótico en el interior celular. La circunstancia más evidente por la que esto acontece en Gram-negativas es por la dificultad del antibiótico para atravesar la membrana externa de la bacteria en cantidad suficiente como para ejercer su acción, algo que sucede en el caso del glicopéptido vancomicina [35]. La rigidez de esta estructura, rica en lipopolisacáridos (LPS), representa también una barrera que obstaculiza la difusión de muchos otros antimicrobianos, dígase macrólidos, aminoglicósidos, rifampicinas o ácido fusídico [36,37]. Otra alternativa para disminuir la concentración intracelular del antibiótico es a través de las bombas de expulsión múltiple de drogas. Estos sistemas figuran entre los elementos más trascendentales en la resistencia intrínseca de las bacterias Gram-negativas, puesto que promueven la expulsión de un dilatado rango de antibióticos. Se clasifican en varias familias, siendo la *Resistance Nodulation Division* (RND) la mejor caracterizada [38]. Constan de una proteína transportadora ubicada en la membrana interna, una proteína de membrana externa y una proteína de fusión de membrana, que anexiona las dos anteriores [39]. Dentro de las más estudiadas y con mayor preponderancia en la resistencia a antibióticos, es menester citar AcrAB-TolC (*Escherichia coli*) [40], CmeABC (*Campylobacter jejuni*) [41] y MexAB-OprM (*P. aeruginosa*) [42]. El hecho de que el cometido funcional de AcrAB-TolC sea la expulsión de sales biliares y péptidos antimicrobianos del hospedador para facilitar la colonización del intestino [43], apoya la noción de que los genes constituyentes del resistoma intrínseco tienen una función atávica distinta a la de resistir la acción de los antibióticos [20].

Se ha de tener presente que el resistoma intrínseco no está conformado exclusivamente por determinantes de resistencia clásicos, verbigracia las enzimas inactivantes de antibióticos, sino que engloba a su vez elementos básicos de la fisiología bacteriana [20], como la biosíntesis de lípidos [44]. Incluso reguladores globales pueden modular la sensibilidad a drogas, como es el caso de Crc, regulador del metabolismo del carbono en *P. aeruginosa*, cuya pérdida de función torna la bacteria más sensible a una miríada de antibióticos [45]. En resumen, la resistencia intrínseca no es una respuesta específica a la presencia de antimicrobianos; por contra, es una característica derivada del propio metabolismo, fisiología e incluso estructura de la bacteria [46].

1.2.2. Resistencia adquirida

La resistencia adquirida puede resultar de dos mecanismos: mutaciones del ADN -cromosómico o plasmídico [47]-, o transferencia de genes de resistencia entre bacterias, pertenecientes o no al mismo género o especie (Figura 2) [48]. En lo que a mutaciones cromosómicas respecta, el ejemplo más diáfano es la selección de éstas en la propia diana del antibiótico. Dichas mutaciones pueden modificar la estructura de la proteína, llevando a una disminución de afinidad entre ésta y el fármaco. Algunos ejemplos de esta añagaza acaecidos en clínica son las alteraciones en RpoB o RpsJ, que causan una pérdida de afinidad por la rifampicina [49] o las tetraciclinas [50,51], respectivamente.

La resistencia puede también ser adquirida mediante la selección de mutaciones en reguladores de la expresión de genes codificantes de determinantes de resistencia, lo cual abre un piélago de posibilidades. Mutaciones en reguladores de bombas de expulsión múltiple o de enzimas inactivantes de drogas son algunos de los ejemplos más extendidos. Siguiendo este orden, el caso paradigmático de resistencia adquirida mediante bombas es el debido a la mutación de su regulador transcripcional local negativo, que provoca el incremento en la expresión del sistema de bombeo y, consiguientemente, aviva la expulsión de sus sustratos. Los supradichos sistemas de expulsión MexAB-OprM en *P. aeruginosa* y CmeABC en *C. jejuni*, así como MtrCDE en *Neisseria gonorrhoeae*, entre otros, alcanzan mayores niveles de expresión cuando sus respectivos reguladores, MexR, CmeR y MtrR, adquieren mutaciones inactivantes [52-54]. Este aumento del nivel de expresión se refleja en una mayor resistencia a los antibióticos que las bombas expulsan. En contraste con esta vía, y aun siendo una situación, *a priori*, más inusitada, se han plasmado recientemente casos de resistencia adquirida mediante mutaciones en las propias subunidades que configuran los sistemas de bombeo. SmeH en *S. maltophilia* [55], MexY en *P. aeruginosa* [56] o AcrB en *E. coli* [57] son algunas de las proteínas estructurales en las que se han detectado cambios de aminoácidos que pueden optimizar el lugar de reconocimiento del antibiótico, lo que facilita una expulsión más eficiente. En el caso de bombas de expulsión con expresión basal reducida, el efecto de estas mutaciones se vería potenciado por la pérdida de función del represor local, que provocaría un repunte concomitante de dicha expresión. En lo que a las enzimas concierne, el ejemplo canónico vendría dado de nuevo por las β-lactamasas de tipo AmpC. Estas cefalosporinasas suelen tener una expresión basal

exigua [58], pero pueden sobre-expresarse cuando se seleccionan mutaciones en represores directos o indirectos de las mismas, como es el caso de AmpD [59]. Al mismo tiempo, mutaciones en los genes codificantes de las propias enzimas son también un problema acuciante, puesto que les permiten ampliar su espectro de sustratos. A saber, AmpC apenas mostraba actividad otrora contra cefalosporinas de cuarta generación como la cefpiroma y cefepima, pero hoy día estos antibióticos pueden ser hidrolizados por la acción de nuevas variantes de AmpC, como se ha detectado *in vitro* [60,61] y en aislados clínicos de *Klebsiella aerogenes* y *Enterobacter cloacae* [62,63].

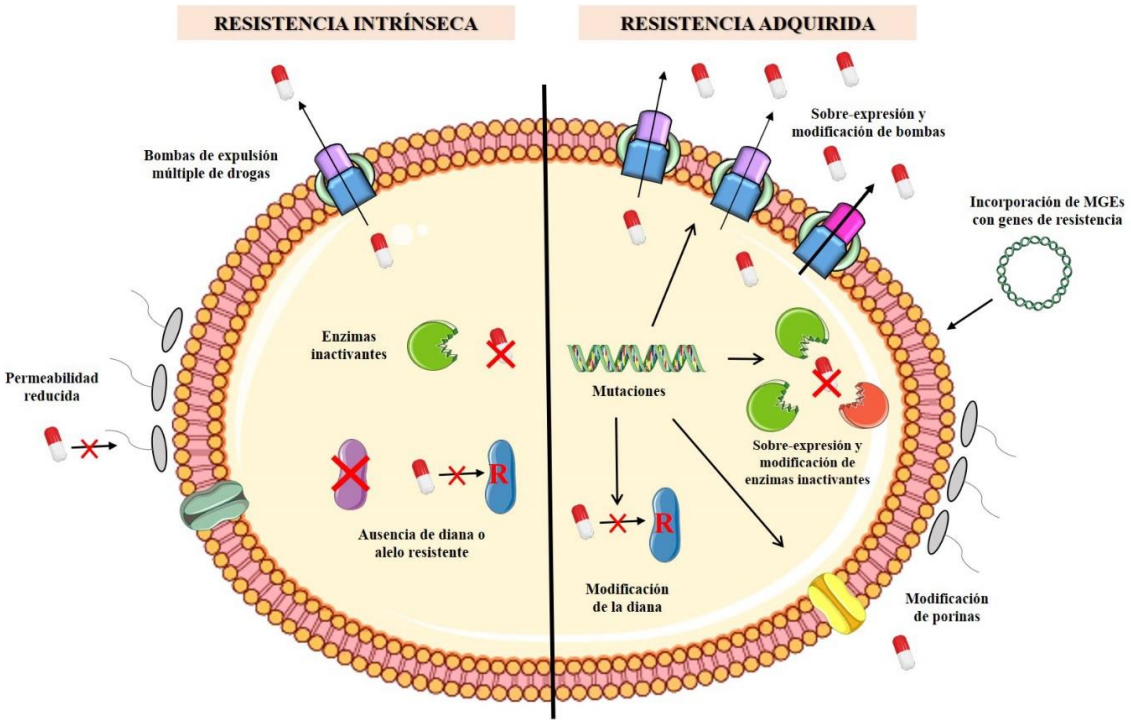

Figura 2. Principales mecanismos de resistencia intrínseca y adquirida en bacterias Gram-negativas. Como primer mecanismo de resistencia intrínseca, el LPS impone una barrera que dificulta el paso del antibiótico al interior celular. Si éste logra franquearla, puede ser expelido mediante bombas de expulsión múltiple de drogas, ser degradado por enzimas inactivantes o verse incapaz de unirse a su diana, al hallarse ésta ausente o codificada por un alelo que produce un blanco resistente. En cuanto a la resistencia adquirida, ésta puede tener lugar mediante la incorporación de MGEs, tales como plásmidos, que portan genes codificantes de determinantes de resistencia. A su vez, las bacterias adquieren resistencia vía mutación a través de varios mecanismos: sobre-expresión de bombas de expulsión múltiple y enzimas inactivantes de drogas (como consecuencia de mutaciones en genes codificantes de reguladores de éstas), modificación de la diana del antibiótico o cambios en la estructura de las porinas, entre otros.

Es de recibo mencionar también las mutaciones que resultan en variaciones en la permeabilidad de membrana, como las que modifican la expresión o estructura de las porinas, o directamente las eliminan [64]. Es el caso de la menor expresión de *phoE* en *K. pneumoniae* [65], o la dispar estructura de OmpF en *K. aerogenes* [66], ambas situaciones asociadas a la adquisición de resistencia en estas bacterias.

A imagen y semejanza de la resistencia intrínseca, la resistencia adquirida puede originarse por mutaciones situadas en *loci* que, en principio, no guardan relación con

determinantes de resistencia clásicos. Mutaciones en *agr* (codificante de un regulador de virulencia en *S. aureus*) [67] o en *pilQ* (codificante de una proteína de *pili* tipo IV en *P. aeruginosa* y *N. gonorrhoeae*) [68,69], propician resistencia a una heterogénea pléyade de antibióticos, por lo que ratifican esta posibilidad.

El otro gran bloque de resistencia adquirida comprende la incorporación de genes de resistencia, que pueden formar parte de casetes contenidos en integrones [70] o en MGEs como plásmidos [71], transposones [72], bacteriófagos [70], o incluso hallarse en el ADN derivado de células bacterianas muertas [48]. Su transferencia puede darse vía transformación o HGT. Entre los casos más preocupantes en la actualidad se encuentran la transferencia de plásmidos que alojan β-lactamasas de amplio espectro [73]; o las islas genómicas características de *Salmonella enterica*, poseedoras de un integrón provisto de numerosos genes de resistencia [74]. A pesar de que esta faceta de la resistencia adquirida es una fuente ubérrima de diseminación y emergencia de la resistencia a los antibióticos en muchas bacterias, no será abordada en esta tesis.

1.2.3. Resistencia fenotípica

Este tercer y último tipo de resistencia a los antibióticos, denominado resistencia fenotípica, adaptativa o transitoria, se define como la disminución de la sensibilidad a un antibiótico derivada de una modificación temporal de la fisiología bacteriana. No conlleva cambios genéticos, por lo que no es heredable, y revierte si la señal o condición ambiental que la indujo desaparece [75]. Tradicionalmente, la situación enmarcada por antonomasia en esta categoría era la persistencia -por la que una subpoblación bacteriana desarrolla un estatus quiescente y refractario a la acción antibiótica [76]-, que puede ocurrir durante el crecimiento en biopelículas [77]. Sin embargo, también han de tenerse en consideración los casos de inducción de un fenotipo resistente por señales y/o condiciones que pueden encontrarse durante el tratamiento antibiótico de infecciones, ya que pondrían en entredicho su eficacia [78]. Algunas de éstas pueden ser el estrés oxidativo o nitrosativo, la temperatura, el modo de crecimiento, moléculas producidas por el hospedador (péptidos catiónicos, ácidos grasos o sales biliares) o incluso los propios antibióticos [79-82].

Los ejemplos de resistencia transitoria reportados hasta la fecha no son tan cuantiosos como los de los dos epígrafes anteriores, pero pueden resaltarse algunos. Primeramente, se han descrito prolijamente bastantes casos de inducción de la expresión de bombas de expulsión por moléculas y condiciones variopintas, lo que puede conducir a fenotipos MDR. En particular, en *P. aeruginosa*, *mexCD-oprJ* y *mexAB-oprM* cuentan con inductores utilizados en práctica clínica, como el anestésico procaína o la amikacina, respectivamente [83]. De guisa similar, a la inducción de los genes que codifican los sistemas SmeDEF y SmeVWX de *S. maltophilia* se han adscrito flavonoides derivados de plantas [84] y el biocida triclosán [85], en el primer caso; y vitamina K3 y antibióticos [81,86], en el segundo.

En esta línea, la existencia de β-lactamasas inducibles (i. e., AmpC) por determinados β-lactámicos en bacterias Gram-negativas supone un obstáculo más para la consecución exitosa del tratamiento de las infecciones que producen [59]. Asimismo, se ha documentado que modificaciones temporales de la carga de membrana en respuesta a concentraciones extracelulares limitantes de Mg^{2+} y Ca^{2+} pueden concitar resistencia fenotípica a péptidos catiónicos y polimixinas en *P. aeruginosa* y *S. enterica* [87-89].

Como corolario, existen otros ejemplos, véase la modulación en la expresión de porinas de *E. coli* con motivo de cambios ambientales [90,91], la producción variable de vesículas de membrana externa -que pueden retener algunos antibióticos como los péptidos catiónicos [75]-, o el caso particular de la heterorresistencia, que describe la situación en la que una población bacteriana contiene subpoblaciones resistentes a antibióticos de manera transitoria, sólo mantenidas por la presión selectiva del antimicrobiano [92]. Ha de aseverarse que este último caso no se encuadra rigurosamente en el cajón de sastre de la resistencia fenotípica, puesto que es debida a cambios genéticos -aunque inestables-, eminentemente amplificaciones en tándem de ciertas regiones cromosómicas [93].

1.3. Efectos de la adquisición de resistencia en la fisiología bacteriana

Tal y como se ha entrevisto en la descripción de los tipos de resistencia bacteriana, la adquisición de ésta suele implicar alteraciones en sistemas que a menudo poseen funciones varias en el entramado de la fisiología celular, lo que provoca efectos colaterales en la misma. Entre ellos, los casos más sobresalientes son el coste de *fitness* y la resistencia cruzada y sensibilidad colateral.

1.3.1. Coste de *fitness* y mecanismos compensatorios

Tiempo ha que se conoce que la adquisición de resistencia a los antibióticos conlleva una serie de cambios metabólicos en las bacterias que pueden dar lugar a una pérdida de competitividad, fenómeno conocido como coste de *fitness* [94,95]. Este concepto indica que los microorganismos han de pagar una suerte de gravamen pleiotrópico al adquirir resistencia, específicamente cuando ésta implica cambios en elementos fundamentales para la fisiología bacteriana [96]. De esta manera, sus efectos se hacen notar principalmente en una reducción de la tasa de crecimiento comparativamente a la cepa silvestre sensible, aunque también se han hallado casos de alteraciones en la formación de biopelículas [97] o la virulencia [98]. Por ello, el coste de *fitness* es un parámetro clave que determina la evolución y persistencia de la resistencia a los antibióticos.

La hipótesis más arraigada en el campo es que el coste de *fitness* podría depender de si la resistencia se adquiere vía mutación o mediante la incorporación de MGEs, ya que el impacto de ambos eventos en la fisiología bacteriana difiere. En el primer caso, el coste ocurre a raíz de la repercusión fisiológica que entraña la mutación de ora genes codificantes de dianas y/o de transportadores de los antibióticos, ora genes codificantes de reguladores de la expresión de elementos de detoxificación (bombas de expulsión

MDR o enzimas inactivantes), ora genes que codifican reguladores globales (MexT o AmpR en *P. aeruginosa*); todos ellos muy conservados y de acreditada importancia en la fisiología bacteriana. Por tanto, su mutación podría trastocar el metabolismo de la bacteria [99-101]. En el caso de la HGT, una visión clásica sugiere que el coste es el resultado de las exigencias fisiológicas que conllevan la replicación, transcripción y traducción de los genes adquiridos [96].

Una vez se tuvo noción de la existencia del coste de *fitness*, por el cual una cepa silvestre sobrepujaría a otra resistente en ausencia de antibiótico, se propuso la rotación cíclica de antibióticos [102]. Infaustamente, la aplicación de esta rotación no tuvo el éxito esperable [103], y pronto comenzaron a detectarse múltiples excepciones a la asunción en que se basaba, en las que la resistencia se revelaba como neutral o incluso beneficiosa para el *fitness* bacteriano. Algunos de los casos descritos de mutaciones de resistencia que no suponen coste alguno son la sustitución K42R en la proteína ribosomal S12 de *E. coli*, *Mycobacterium tuberculosis* y *S. enterica*. Otras mutaciones incluso producen una mejora del *fitness*, como C257T en *gyrA* de *C. jejuni* [104], si bien en este caso el efecto beneficioso es dependiente de la cepa que contiene el cambio. Esto ejemplifica la situación en la que el coste asociado a ciertas mutaciones de resistencia depende del contexto genético en el que se adquieren, lo que apunta a la relevancia de las relaciones de epistasia en el *fitness* bacteriano [105,106]. Este factor será desbrozado más adelante.

El efecto dependiente del contexto genético está relacionado, ocasionalmente, con el hecho de que el coste de *fitness* aparejado a la adquisición de resistencia puede compensarse mediante mutaciones secundarias. Estas mutaciones pueden revertir el efecto de aquella que causaba el coste de *fitness*, ya sea reemplazando la función que se hallaba comprometida, reduciendo la necesidad de esa función o restableciendo la actividad de la proteína afectada [96]. Esta evolución compensatoria ha sido observada en numerosos estudios, siendo uno de los más renombrados el de la resistencia a isoniazida de *M. tuberculosis* debido a mutaciones en *katG*, cuyo lastre pleiotrópico se ve amainado con cambios genéticos sitos en *aphC* [107]. Es pertinente hacer ver que la selección de mutaciones compensatorias es dependiente del ambiente, de modo que la citada epistasia entre éstas y las mutaciones de resistencia también lo es. Otro sendero algo menos consuetudinario para compensar el coste de *fitness* es la adquisición de mutaciones de resistencia secundarias que puedan contrapesar el coste asociado a mutaciones de resistencia primarias [108]. La interacción resultante puede causar que la resistencia a un antibiótico sea superior en un mutante previamente resistente a otra droga [109]. Esta situación ha sido detectada en mutantes de *P. aeruginosa* resistentes a estreptomicina, que portan la sustitución K88E en RpsL, cuyo coste de *fitness* puede ser compensado por mutaciones secundarias de resistencia a rifampicina [110,111].

Además de mediante mutaciones secundarias, la compensación puede darse gracias a amplificaciones génicas, algo cotejado en mutantes de *S. enterica* resistentes a actinonina [112]. Y quizá la compensación más epatante provenga de la posibilidad de remitir el coste de *fitness* manteniendo el material genético intocado. Se trata de

reorganizaciones metabólicas que aligeran esa carga mediante variaciones en la expresión de genes que pueden estar involucrados en otros procesos celulares. Ha sido descrita en mutantes de *P. aeruginosa* que sobre-expresan bombas de expulsión MDR [113], y en micobacterias resistentes a la capreomicina [114]. Nótese que el coste de *fitness* originado por eventos de HGT también cuenta con mecanismos de estabilización [115], pero no se incidirá en ello en esta tesis.

1.3.2. Resistencia cruzada y sensibilidad colateral

Otro de los efectos propiciados por la adquisición de resistencia a un determinado antibiótico puede ser el incremento de la resistencia o de la sensibilidad a otros antibióticos desemejantes al primero, fenómenos que se conocen, ya desde los años 50, como resistencia cruzada y sensibilidad colateral, respectivamente [116].

Los mecanismos que subyacen en la resistencia cruzada pueden variar; no obstante, la sobre-expresión de bombas de expulsión múltiple de drogas es considerada como una de las mayores responsables de su emergencia, dada la variada hornada de sustratos que expelen [117]. No están solas en este ámbito: la resistencia cruzada entre antibióticos de la misma familia estructural también puede surgir a partir de mutaciones en la diana común que comparten [118]. Además, mutaciones compensatorias del coste de *fitness* [119] y alteraciones en genes que, si bien son dianas específicas de un antibiótico, pueden conducir a modificaciones en las rutas de respuesta a estrés global al medrar su expresión [120,121], serían otras de las causas más frecuentes de resistencia cruzada entre antimicrobianos de naturaleza heteróclita.

Por su parte, aunque hay estudios al respecto [122], los mecanismos moleculares que explican la sensibilidad colateral son algo menos conocidos, soliéndose achacar a una consecuencia pleiotrópica de las mutaciones de resistencia [123,124] y a la función de los genes que las sufren [125]. Un ejemplo estudiado de mecanismo molecular de sensibilidad colateral es la reducción del potencial de membrana, que explica el nexo existente entre la resistencia a aminoglicósidos y la hipersensibilidad a β-lactámicos en *E. coli* [119,126]. En este caso, la mayor resistencia a aminoglicósidos, como consecuencia de una menor entrada de los mismos, va asociada a un aumento en la sensibilidad a β-lactámicos, porque su expulsión mediante bombas MDR es dependiente del potencial de membrana [126,127]. Dicho esto, la trascendencia de la sensibilidad colateral radica realmente en lo siguiente: *a priori*, este *trade-off* evolutivo podría explotarse para eliminar bacterias resistentes mediante el uso de antibióticos para los que hayan adquirido sensibilidad colateral, algo que se ha planteado desde la perspectiva de la terapia combinada [128,129] o la alternancia de parejas de antimicrobianos [123,130]. Es más, el estudio reciente de los fenotipos de sensibilidad colateral ha establecido mimbres desde los que la utilidad de los tratamientos secuenciales podría verse impulsada [131].

No obstante, la aplicabilidad de estas aproximaciones a la clínica adolece todavía de cierta robustez. La reproducibilidad, durabilidad y, sobre todo, conservación evolutiva

de los patrones de sensibilidad colateral entre especies bacterianas o entre dispares cepas de la misma especie, son factores a tener en cuenta que no han recibido tanto pábulo como debieran [121,132-135]. Por tanto, el hallazgo de trayectorias evolutivas conservadas hacia la resistencia a un antibiótico en diferentes contextos genéticos -situación más próxima a la realidad clínica que el común uso de cepas modelo-, que estuviera asociada a un mismo patrón de sensibilidad colateral, supondría un espaldarazo notable a la posible implementación de los antedichos tratamientos secuenciales *in vivo*.

1.4. Predicción de la resistencia a los antibióticos

¿Es posible predecir la resistencia a los antibióticos? A sabiendas de la naturaleza estocástica de la evolución [136], así como la colosal variedad de determinantes de resistencia conocidos y los todavía ignotos, la respuesta debería ser un palmario no. Empero, hoy día se ha recabado el suficiente número de evidencias y desarrollado técnicas de predicción lo bastante atinadas como para orlar con un tenue nimbo de esperanza esta posible prognosis, que supondría un aliado inestimable en la perenne liza contra la amenaza de la resistencia a los antibióticos [137]. La susodicha predicción es factible porque existen diversos factores que restringen la evolución de la resistencia, haciendo que algunas rutas evolutivas tengan una mayor probabilidad de ser seleccionadas cuando un antibiótico se aplica, ya sea por el nivel de resistencia al que conducen o por su bajo coste de *fitness* anejo [138].

Las metodologías usadas para abordar esta predicción abarcan desde modelos bioinformáticos y de diagnóstico molecular [139-141], hasta procedimientos *in vitro* [142] e *in vivo* [143]. En esta tesis, se han utilizado dos técnicas predictivas ampliamente extendidas, que nutren de información sobre resistencia intrínseca y adquirida, respectivamente: el escrutinio de genotecas de inserción y los ensayos de evolución adaptativa en laboratorio (*Adaptive Laboratory Evolution*: ALE).

1.4.1. Predicción de la resistencia intrínseca: escrutinio de genotecas de inserción

La identificación de elementos cromosómicos que contribuyen a la reducción de la sensibilidad a antibióticos en bacterias (o lo que es lo mismo, miembros del resistoma intrínseco [20]) reviste gran interés, tanto para el posterior estudio de esos mecanismos, como para predecir su rol en la resistencia *in vivo*. Para ello, uno de los métodos más utilizados y fructíferos ha sido el escrutinio de genotecas de mutantes de inserción. Así, se han sucedido una pingüe cantidad de estudios a este respecto, en bacterias de notorio interés clínico como *P. aeruginosa* [25,44,144-146], *E. coli* [23] y *S. aureus* [147,148], u otras menos ínclitas como *Acinetobacter baylyi* [149].

Por añadidura, el escrutinio de genotecas de inserción puede desembocar en el hallazgo de genes cuya inactivación conlleva cambios en la virulencia de la bacteria [150,151]. Por si fuera poco, y pese a no ser información ligada al resistoma intrínseco, es útil

documentar los genes que, inactivados, ocasionan una atenuación en la sensibilidad a ciertos antimicrobianos, porque ese hecho apuntaría a su posible papel en la resistencia adquirida, en este caso vía mutaciones inactivantes.

Siendo rigurosos, huelga decir que este método predictivo dista de ser perfecto. En primer lugar, subestima el tamaño del resistoma intrínseco, ya que sólo identifica genes no esenciales (aquellos que han podido ser inactivados en los mutantes de inserción). En segunda instancia, se limita a evaluar el fenotipo de sensibilidad cuando un gen se halla completamente inactivo, arrumbando las mutaciones de inactivación parcial o las de ganancia de función. Es más, existe otro matiz a entresacar en esta técnica: algunos de estos análisis llevados a cabo con un antibiótico como representante de su familia estructural han asumido que los resultados obtenidos podrían ser extrapolables al resto de miembros de dicha familia [25,146,148], pero tal supuesto no ha sido analizado en profundidad. Ergo, esta cuestión será examinada en esta tesis, mediante la determinación de la sensibilidad de mutantes de inserción de *P. aeruginosa* -extraídos de un escrutinio en el laboratorio y de publicaciones pretéritas [25,44]- frente a cuatro aminoglicósidos. De esta forma y manera, hemos elucidado si la inactivación de un gen concreto que propicia una alteración en la sensibilidad a un antibiótico, puede causar el mismo efecto (sensibilidad o resistencia) frente a otros antimicrobianos de la misma familia.

1.4.2. Predicción de la resistencia adquirida: ALE y factores que la constriñen

Los escrutinios de genotecas de inserción detectan determinantes de la resistencia intrínseca, y aquellos cuya inactivación aumenta la susodicha. Sin embargo, como ya se ha advertido, presentan la traba de no posibilitar el análisis de mutaciones de pérdida de función parcial o de ganancia de la misma. De hecho, no permiten predecir si esos mecanismos de resistencia se seleccionarían con mayor preponderancia durante una terapia con antibiótico, porque para ello sería necesario tener en consideración el coste de *fitness* de dichos mecanismos. Así, entra en escena el siguiente método predictivo a pormenorizar. Con el fin de profundizar en el estudio la evolución bacteriana, Richard Lenski comenzó, a finales de los 80, sus ensayos de evolución experimental, conocidos como experimentos ALE [152]. Dada la velocidad de crecimiento de las bacterias y el tamaño ciclópeo de sus poblaciones, esta metodología allana el camino hacia el estudio de la evolución de los microorganismos en presencia de distintas presiones selectivas, como puede ser un antibiótico [153-156], en periodos de tiempo muy cortos. Esto, añadido al desarrollo de las técnicas de secuenciación de genomas bacterianos, ha hecho despuntar el potencial predictivo de los ensayos ALE.

En términos generales, la evolución experimental en presencia de concentraciones letales de antibiótico consiste en la exposición de una determinada población bacteriana a concentraciones de antimicrobiano lo bastante elevadas como para inhibir su crecimiento, a la par que lo bastante permisivas como para posibilitar la selección de mutantes espontáneos [155]. Este proceso se prolonga durante un tiempo concreto,

normalmente el necesario para dar lugar a cientos o miles de generaciones, con objeto de favorecer la selección de los mutantes mejor adaptados. Simultáneamente, la concentración de antibiótico puede incrementarse a lo largo del tiempo -si se desea favorecer la selección de un mayor número de mutaciones-, lo que se espejará en el aumento de resistencia que presentarán las poblaciones (Figura 3) [157]. Una vez consumada la evolución *per se*, se procede a evaluar la sensibilidad al antibiótico en cuestión, así como a analizar las mutaciones presuntamente responsables de la adquisición de la resistencia mediante la secuenciación de los genomas de poblaciones completas o de clones individuales aislados de las mismas. Aparte, la posibilidad de reconstruir el orden de aparición de las mutaciones a lo largo de la evolución adaptativa nos otorga la opción de vislumbrar patrones evolutivos que hagan de la predicción algo factible [138,157].

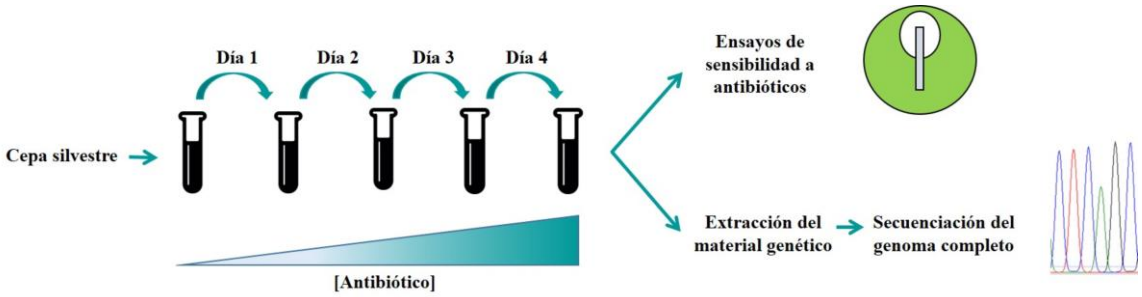

Figura 3. Diagrama que expone el procedimiento ALE en presencia de antibiótico. Un determinado porcentaje de la población de la cepa bacteriana es transferido de forma circadiana a medio de cultivo nuevo con antibiótico como presión selectiva, hasta un número estipulado de generaciones. Puede incrementarse la concentración de antibiótico cada cierto periodo de tiempo para seleccionar combinaciones de mutaciones. Al final del proceso, se cuantifica el nivel de resistencia de la población bacteriana y se indaga en las causas genéticas de ésta mediante la secuenciación de su genoma.

No obstante, la validez de la evolución experimental como método de predicción de emergencia de resistencia frente a agentes antimicrobianos con relevancia clínica ha sido ocasionalmente cuestionada, dadas las diferencias que existen entre las condiciones de crecimiento bacteriano *in vitro* y las de una situación de infección. Empero, aunque es innegable que se precisan más estudios comparativos entre las mutaciones detectadas en una y otra vertiente [158], la capacidad de esta técnica para predecir mutaciones de resistencia presentes *in vivo* ha sido probada frente a colistina, tigeciclina, β-lactámicos, quinolonas o aminoglicósidos, entre otros [69,159-161]. Es más, hay trabajos que respaldan la avenencia entre las esferas *in vivo* e *in vitro* en base al análisis del *fitness* de mutantes de origen clínico tras ser reconstruidos en laboratorio [108,162].

Llegados a este punto, y teniendo presente el elevado número de genes que podrían contribuir a la adquisición de resistencia por mutación en una población bacteriana: ¿qué factores constriñen su evolución, de modo que lo que podría ser al albur está a veces dotado de un cierto grado de conservación? Los cuatro más egregios son los siguientes: el impacto de cada mutación en el *fitness* relativo, el nivel de resistencia alcanzado (dependiente de la naturaleza y nivel de expresión del determinante de

resistencia), la frecuencia de mutación (subordinada al tamaño poblacional) y la presión de selección, siendo quizá los dos primeros los más determinantes [138]. No obstante, existen otros elementos que también restringen la evolución, como los cuellos de botella poblacionales (dependientes del tamaño del inóculo), la interferencia clonal (competición entre mutantes con fenotipo seleccionable, que depende en última instancia del *fitness*), la evolución compensatoria (mutaciones que restauran el *fitness* depauperado), los fenómenos de sensibilidad colateral (por los que la adquisición de una mutación de resistencia a una droga coarta el crecimiento en presencia de otra dispar) y, por último, pero no menos importante, la epistasia (por la que el nivel de resistencia o *fitness* asociado a una mutación depende del contexto genético en que se selecciona) (Figura 4) [124,125,163-166]. De entre esta gama de factores que compelen la evolución de la resistencia y que se encuentran estrechamente relacionados entre sí, dos de ellos serán descritos a continuación en mayor profundidad, a razón de su importancia en el transcurso de esta tesis: la presión de selección y la epistasia.

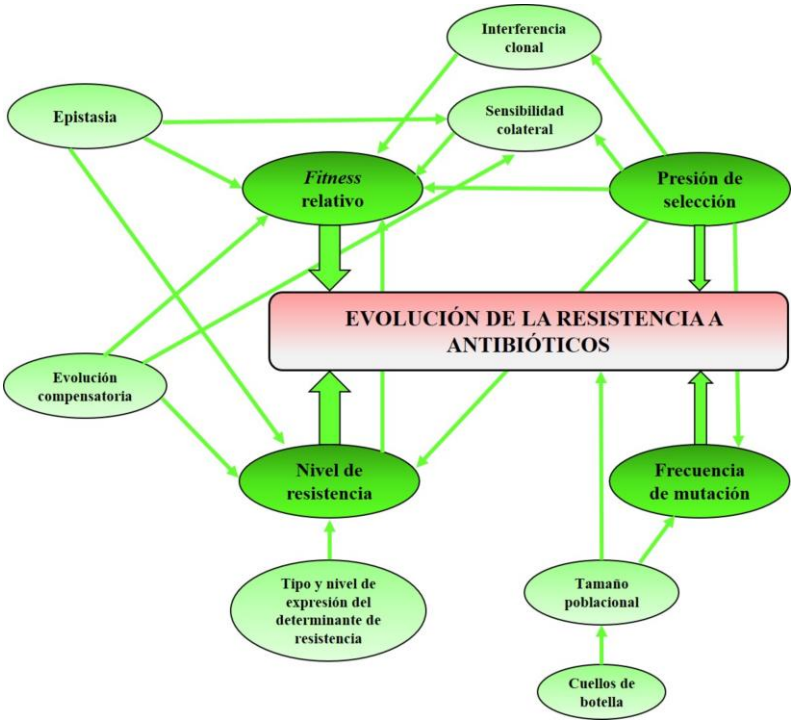

Figura 4. Mapa conceptual que reúne los factores que determinan la evolución de la resistencia a los antibióticos por mutación, así como las interacciones entre ellos. Cuatro son los factores capitales en esta urdimbre: el *fitness* relativo del mutante resistente, la presión de selección, el nivel de resistencia que confiere el mecanismo adquirido y la frecuencia de mutación. A la sazón, otros agentes ejercen su influencia sobre ellos, como pueden ser la epistasia, la evolución compensatoria o el tamaño poblacional.

1.4.2.1. Presión de selección

El grado y tipo de presión de selección que sufre una población bacteriana es uno de los factores más determinantes a la hora de aherrojar la singladura evolutiva hacia la resistencia a antibióticos. Existen numerosos datos acerca de su influencia sobre la frecuencia de mutación [167], el *fitness* relativo [119], el nivel de resistencia alcanzado [168], la resistencia cruzada o la sensibilidad colateral [119]. Esta influencia tiene un efecto

contraintuitivo en algunos casos: una baja presión de selección puede dar lugar a elevados niveles de resistencia; así como a una menor variedad de mutantes que la propiciada por una presión de selección mayor, a pesar de que los mutantes de bajo nivel de resistencia sólo serían seleccionables bajo una presión de selección leve [119,168].

En general, el efecto que ejerce la presión de selección en la evolución de la resistencia se manifiesta al variar su régimen de aplicación o calibre [169], siendo probablemente el caso más arquetípico y estudiado el de las concentraciones subletales de antibiótico. Tradicionalmente se ha considerado que la ventana de selección de mutantes resistentes se ubica en un rango de concentraciones de antibiótico flanqueado por la CMI de una cepa silvestre y su concentración preventiva de mutantes (CPM), siendo esta última la concentración que previene la aparición de mutantes resistentes con una sola mutación (Figura 5) [170-172]. Sin embargo, estudios recientes han desmentido este canon, probando que concentraciones subletales de antibiótico (inferiores a la CMI) son capaces de seleccionar mutantes resistentes, incluso de elevado nivel de resistencia [168,173]. Conforme a esto, se han descrito múltiples efectos que estas concentraciones pueden ocasionar en las poblaciones bacterianas, como es la inducción de una mayor frecuencia de mutación [174] o la selección de mutaciones de resistencia con un ínfimo coste de *fitness* [175].

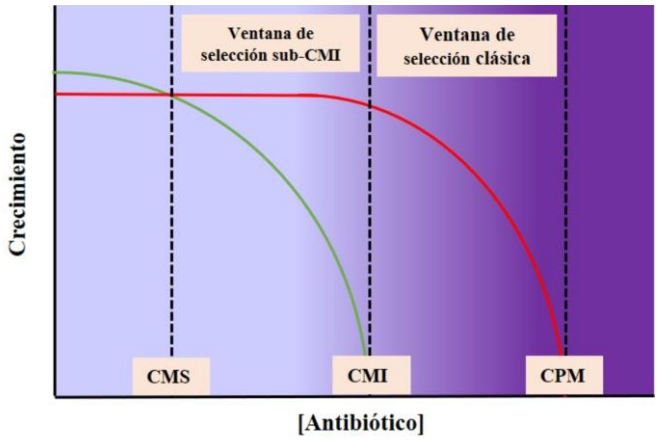

Figura 5. Gráfico que muestra las dos ventanas de selección de resistencia a los antibióticos. La clásica abarca desde la CMI de la cepa silvestre hasta la CPM. Por su parte, la ventana de selección sub-CMI se sitúa entre la concentración mínima de selección (CMS), que depende de la naturaleza del antibiótico y del *fitness* en presencia del mismo, y la CMI. Curva verde: crecimiento de cepa sensible. Curva roja: crecimiento de cepa con una mutación de resistencia. Imagen modificada de [175].

Bajo estos descubrimientos subyace un problema que globaliza más si cabe la amenaza de la resistencia a los antibióticos: las concentraciones subletales de antibiótico no sólo se encuentran en clínica (en tejidos de accesibilidad limitada para el fármaco o como consecuencia de tratamientos incompletos) [172], sino que también proliferan en entornos naturales, véase ríos, lagos, lodos, aguas residuales, etc [176,177]. Por ende, se impone la necesidad de adentrarse en el estudio de la selección de resistencia a antibióticos bajo presiones de selección sub-CMI, con objeto de identificar el rango de concentraciones de cada antimicrobiano que selecciona resistencia, y los posibles casos de resistencia cruzada y sensibilidad colateral que lleven aparejados. Tal iniciativa, que aunque ya

abordada, es todavía incipiente [119,175,178], reviste especial urgencia en el caso de patógenos de origen ambiental como *P. aeruginosa*, dado que pueden colonizar hábitats que contengan concentraciones muy variadas de drogas.

1.4.2.2. Epistasia

La epistasia define el fenómeno por el que diferentes genes interaccionan a la hora de expresar un determinado carácter fenotípico. Un ejemplo específico de la misma sería la influencia que ejerce el contexto genético sobre el efecto fenotípico de una mutación en un gen concreto [179]. En el tema que nos ocupa, esta situación puede hacer fluctuar radicalmente el nivel de resistencia y/o el *fitness* relativo de un mutante resistente individual, en dependencia de la presencia o ausencia de otra/s mutación/es [133,180]. Si el fenotipo adquirido (resistencia o modificación del *fitness*) es superior al de cada mutante por separado, se trataría de epistasia positiva; mientras que, si éste es inferior al del mutante individual, se trataría de epistasia negativa.

Así, la epistasia ejerce un efecto marcado sobre la evolución de la resistencia a antibióticos: el acúmulo paulatino de mutaciones en diferentes *loci* introduce cortapisas que restringen el, *a priori*, inmenso telar de trayectorias evolutivas hacia la resistencia [159,163,179]. Esto recalca la importancia del orden en que se adquieren las mutaciones, puesto que la selección o no de éstas dependerá de la presencia de las precedentes, concepto conocido como contingencia [181,182]. Por ello, la epistasia puede tanto dificultar la predicción de las rutas evolutivas más probables hacia la resistencia, ya que éstas mudarían en función del contexto genético, como reducir la cantidad de las mismas, ya que la contingencia limita el número de mutaciones potencialmente seleccionables.

Al hilo del tema que encabeza la sección, los ensayos ALE se han consagrado en algún caso al estudio del papel de la epistasia en el modelado de las rutas evolutivas hacia la resistencia [183,184], mas el objetivo de estos estudios suele estribar en las hipotéticas interacciones que puedan ocurrir entre mutaciones que confieren resistencia a antibióticos [109,133,179,185,186]. Un campo no tan feraz por el momento, y cuyo busilis se enfatizará en esta tesis, es el que contempla la epistasia entre mutaciones de resistencia y de adaptación al ambiente, dos facciones que se han revelado como interconectadas [46]. El interés de este abordaje gravita sobre el hecho de que muchos patógenos humanos, como es el caso de *P. aeruginosa* durante la infección crónica en pacientes con fibrosis quística (FQ), confluyen en la adquisición de mutaciones eco-adaptativas semejantes, pero su abanico de mutaciones de resistencia posibles puede divergir [187]. El cómo esas premisas genéticas puedan delimitar la evolución de la resistencia sería un conocimiento de gran utilidad, por lo que ha sido tema de estudio en esta tesis.

1.5. *Pseudomonas aeruginosa*

P. aeruginosa es un bacilo Gram-negativo anaerobio facultativo, cuya versatilidad metabólica le dota de ubicuidad [188], ergo es capaz de colonizar una plétora de hábitats.

Entre éstos se encuentran el suelo [189,190], las aguas residuales [191] o incluso el petróleo [192], siendo los lugares vinculados a la actividad humana su nicho predilecto [193]. Asimismo, se trata de un patógeno oportunista con un rango de hospedadores que abarca plantas [194,195] y animales [196,197], incluyendo la especie humana [198].

Al respecto de su rol como patógeno, *P. aeruginosa* es una de las causas principales de las infecciones de índole nosocomial, i. e. infecciones agudas respiratorias o bacteriemias. Asimismo, puede infectar crónicamente a pacientes aquejados de FQ [199], enfermedad pulmonar obstructiva crónica (EPOC) [187], cáncer [200], SIDA [201] o quemaduras [202]. En definitiva, propende a colonizar pacientes inmunocomprometidos y/o con patologías basales, lo que la erige como un artífice incuestionable de la mortalidad en las Unidades de Cuidados Intensivos (UCI) [203].

Este hecho se ve agravado por algunas de las características que presenta esta bacteria, entre las que destacan su capacidad para formar biopelículas -algo particularmente peliagudo en catéteres, prótesis o el propio pulmón [204-206]-; o su elevada resistencia intrínseca a un sinnúmero de antibióticos [21,144,145,207], al cual se circunscriben incluso antimicrobianos de reciente aparición, verbigracia la glicilciclina tigeciclina [208]. Por si fuera poco, *P. aeruginosa* puede adquirir resistencia a antibióticos, bien por mutación -algo que acaece frecuentemente durante las infecciones crónicas [209,210]-, bien mediante la incorporación de genes de resistencia a antibióticos vía HGT [211].

En consecuencia, estas particularidades hacen a *P. aeruginosa* acreedora de engrosar las anteriormente citadas agrupaciones ESKAPE [16] y TOTEM [17], en las que figuran las bacterias multirresistentes de mayor relevancia global en la actualidad.

1.5.1. Mecanismos de resistencia a los antibióticos en *P. aeruginosa*

La resistencia intrínseca de esta bacteria pivota sobre varios pilares: su baja permeabilidad de membrana (varios órdenes de magnitud menor que la de *E. coli*) [212,213], la gran cantidad de bombas de expulsión múltiple de drogas que posee [214] y la producción de enzimas inactivantes de antibióticos [215,216].

En lo que a los sistemas de bombeo concierne, si bien se han descrito hasta la fecha 12 miembros de la familia RND presentes en *P. aeruginosa* [217], son 4 los que cumplen un papel más prominente en la resistencia a antibióticos: MexAB-OprM, MexCD-OprJ, MexEF-OprN y MexXY-OprM [42,218-220], siendo el primero y el último los únicos que contribuyen a la resistencia intrínseca, mas todos ellos pudiendo expulsar una gran miscelánea de antibióticos (Tabla 1). Por su parte, entre las enzimas inactivantes de antibióticos brillan con luz propia las β-lactamasas, siendo su máximo exponente la codificada por *ampC*; y las enzimas inactivantes de aminoglicósidos: a saber, aminoglicósido acetiltransferasas, fosfotransferasas y nucleotidiltransferasas [221].

Tabla 1. Bombas de expulsión múltiple de drogas con relevancia clínica en *P. aeruginosa*.

Bomba	Reguladores	Sustratos	Resistencia	Referencias
MexAB-OprM	MexR, NalD, NalC, Mdr1, Mdr2, CpxR	β-lactámicos (excepto imipenem), quinolonas, macrólidos, tetraciclinas, cloranfenicol	Intrínseca Adquirida Fenotípica	45, 205-209
MexCD-OprJ	NfxB, EsrC	Penicilina, cefepima, cefpiroma, meropenem, quinolonas, macrólidos, tetraciclinas, cloranfenicol	Adquirida Fenotípica	76, 210, 211
MexEF-OprN	MexT, MexS	Carbapenemas, quinolonas, cloranfenicol	Adquirida Fenotípica	212-214
MexXY-OprM	MexZ, ParRS, PA2572	Penicilina, cefepima, cefpiroma, meropenem, quinolonas, macrólidos, tetraciclinas, cloranfenicol, aminoglicósidos	Intrínseca Adquirida Fenotípica	215-217

La adquisición de mutaciones en represores de la expresión de los determinantes de resistencia antecitados desemboca en su sobre-expresión. Por ejemplo, el sistema de bombeo codificado por *mexAB-oprM* presenta un incremento de expresión a raíz de mutaciones en el gen correspondiente a su regulador local MexR [52] o a otros reguladores secundarios, como NalC y NalD [222,223], algo que se ha detectado con asiduidad en clínica [224,225]. La sobre-expresión de *mexCD-oprJ*, por mutaciones en el gen que codifica su regulador NfxB, y de *mexXY-oprM*, por mutaciones en *mexZ*, son a su vez casos que menudean [225,226]. Por otro lado, han sido descritas mutaciones en genes de represores directos o indirectos de la expresión de *ampC*, llámense AmpR o AmpD. Ítem, no es extraño encontrar mutaciones de pérdida de función en genes cuyo desempeño se relaciona con el reciclaje del peptidoglicano (*mpl*, *dacB*) [227-229] -que también elevan la actividad β-lactamasa-; o en genes que codifican dianas de los antibióticos, como *gyrA*/*gyrB*, propiciando resistencia a quinolonas [230]; o bien en genes codificantes de proteínas de unión a penicilinas (*Penicillin Binding Proteins*, PBPs), como PBP3, causando resistencia a β-lactámicos [142,231].

Finalmente, no se ha de soslayar la posibilidad de que *P. aeruginosa* adquiera mediante HGT genes de resistencia a antibióticos localizados en plásmidos o integrones [232,233]; o desarrolle resistencia fenotípica, ya sea a través de la expresión inducida de las mentadas bombas de expulsión o generando biopelículas [75,234].

1.5.2. Factores de virulencia

P. aeruginosa posee una serie de factores de virulencia que facilitan su proceso infectivo. El listado es luengo y variado: el LPS, el flagelo y *pili* tipo IV -que contribuyen a diversas formas de motilidad: *swarming*, *swimming* y *twitching*, vinculadas a la colonización pulmonar-, proteasas como la elastasa -cuya actividad daña

ciertos tejidos del hospedador-, sistemas de secreción -siendo especialmente relevante en patogénesis el III-, o formación de biopelículas. Es de reseñar que la producción de los determinantes de virulencia puede regularse de modo concertado, tesitura en la que el sistema de señalización por *quorum sensing* (QS) juega un papel neurálgico [235,236].

A tal respecto, el QS es un sistema de comunicación intercelular que depende de la densidad poblacional, y que está basado en la producción de moléculas autoinductoras que activan la expresión de genes codificantes de reguladores maestros, alterando el transcriptoma celular y, a la postre, la fisiología bacteriana [237]. En la bacteria que nos ocupa, el QS es orquestado por tres reguladores, íntimamente interrelacionados: LasR, RhlR y PqsR [238]. Entre sus efectos a nivel fenotípico, se encuentra la regulación de la producción de muchos de los factores de virulencia mencionados, i. e. biopelículas, proteasas [239] o piocianina, una toxina (amén de un pigmento) que puede inducir respuesta ROS en el hospedador [240]. Otro pigmento generado por *P. aeruginosa* es la piomelanina, una molécula que dota de coloración marrón y que es frecuentemente detectada (hasta en un 13%) en aislados clínicos de pacientes con FQ [241]. La hipótesis más aceptada sobre su función pivota en la resistencia que podría conferir al estrés oxidativo generado por los macrófagos, favoreciendo así la persistencia en las infecciones crónicas pulmonares [242].

1.5.3. Estrategias terapéuticas para tratar las infecciones por *P. aeruginosa*

Grosso modo, las infecciones ocasionadas por *P. aeruginosa* son tratadas con aminoglicósidos, especialmente tobramicina, a menudo administrada en aerosoles [243,244]; así como con cefalosporinas (eminentemente ceftazidima) o la combinación de penicilinas con inhibidores de β-lactamasas [245]. Otras elecciones habituales son las fluoroquinolonas (ciprofloxacino), las polimixinas, la fosfomicina, los carbapenemas y el aztreonam [246,247], optándose por uno u otro según la tipología de la infección.

Sin embargo, a fuer de la prolífica resistencia que esgrime este patógeno, en los últimos años se ha impuesto la necesidad de sondear el uso de nuevas combinaciones de antibióticos o el desarrollo de nuevos compuestos, ejemplificados por la plazomicina o el doripenem, entre otros [214,248]. En lo concerniente a las combinaciones, el incipiente empleo de parejas noveles de β-lactámico-inhibidor de β-lactamasas ha demostrado ser efectivo para combatir la muy extendida resistencia bacteriana auspiciada por β-lactamasas de clase A [245,249]. Por otra parte, también se han desarrollado combinaciones eficaces contra otras clases de β-lactamasas, como la dupla ceftazidima-avibactam, que fue aprobada por la FDA en 2015 [250].

El avibactam es un inhibidor de β-lactamasas que posee una potente actividad contra las de clase A, C y algunas de clase D [251], y que, hasta la fecha, ha sido utilizado -en connivencia con la ceftazidima- en casos clínicos críticos: infecciones urinarias e intra-abdominales complicadas, en combinación con metronidazol [252]; si bien se prevé su inclusión inminente en las terapias para erradicar *P. aeruginosa* multirresistentes en

pacientes aquejados de FQ [252]. De ahí el interés en el estudio de los mecanismos de resistencia que este patógeno pueda enarbolar para eludir el efecto de la ceftazidima-avibactam, un ámbito que, hasta hace poco, era rayano en lo ignoto [231,253,254].

En otro orden de cosas, es importante resaltar que *P. aeruginosa* a menudo está presente en superinfecciones asociadas a terapias de infecciones causadas por otros patógenos, lo que contraindica el uso de antibióticos que, si bien puedan ser eficaces contra los otros microorganismos presentes en la infección original, podrían ejercer un efecto nulo contra *P. aeruginosa* o, lo que es peor, seleccionar mutantes resistentes de ésta. Es el caso de la tigeciclina [255-257], glicilciclina para la cual esta bacteria es intrínsecamente resistente [258], entre otras razones, a resultas de su bomba MexXY-OprM, capaz de expulsarla [259]. Ante esta situación, cobra relevancia el ahondar en los efectos que pueda tener el exponer este patógeno oportunista a tigeciclina, en lo que a su resistencia cruzada a otros antimicrobianos de interés clínico se refiere. De esta guisa, se podría mejorar ostensiblemente el diseño de las terapias de estas superinfecciones [256].

Por último, cabe reseñar la importancia que entraña explorar otras alternativas a los antibióticos en el tratamiento de *P. aeruginosa*, a fin de evitar la emergencia de resistencia que periclita su efectividad. Es por ello que también se ha investigado profusamente el desarrollo de compuestos capaces de inhibir la virulencia de este patógeno, ora usados en solitario, ora en terapia combinada con antibióticos [260-262]. No obstante, ni siquiera estos tratamientos se hallan horros de la posibilidad de que emerja resistencia [263]. Por ende, el hallazgo de moléculas que supriman simultáneamente la expresión de factores de virulencia y la resistencia a antibióticos tiene un inmenso interés, si bien aún es un campo al que le resta bastante recorrido [151,264].

En esta tesis nos hemos focalizado en el estudio de la resistencia intrínseca de *P. aeruginosa* a aminoglicósidos, escrutinio de genoteca de inserción mediante; y de su resistencia adquirida a tobramicina, tigeciclina, ceftazidima y ceftazidima-avibactam, ALE mediante. De esta manera, se ha acrecentado el conocimiento sobre los mecanismos de resistencia que esgrime este patógeno frente a estos antimicrobianos y se han hecho predicciones de las trayectorias evolutivas que podrían seleccionarse cuando dichos antibióticos fuesen empleados. Además, los ensayos de ALE nos han permitido escudriñar con mayor realce algunos de los factores que constriñen esa adquisición progresiva de resistencia, principalmente la epistasia, la presión de selección y la sensibilidad colateral.

En cómputo global, como resultado de esta tesis han sido publicados seis estudios en revistas científicas de primer cuartil, en los que se engloban conclusiones, a la par que cuestiones que deberían ser respondidas en un futuro próximo, concernientes a la crítica situación actual en torno a la emergencia y diseminación de la resistencia a los antibióticos y el rol protagónico que *P. aeruginosa* ostenta particularmente en ella.

OBJETIVOS

2. Objetivos

2.1. Estudio de la resistencia intrínseca en *P. aeruginosa*

I. Determinar si el resistoma intrínseco de *P. aeruginosa* para un aminoglicósido dado es común al de otros miembros de su familia estructural.

II. Buscar genes del resistoma intrínseco de *P. aeruginosa* a antibióticos de dispar familia estructural implicados en virulencia, para definir potenciales dianas de coadyuvantes de antibióticos en terapias anti-resistencia y anti-virulencia contra las infecciones debidas a este patógeno.

2.2. Estudio de la resistencia adquirida por mutación en *P. aeruginosa*

I. Analizar la predictibilidad de las trayectorias evolutivas de *P. aeruginosa* en presencia de tobramicina, tigeciclina, ceftazidima y ceftazidima-avibactam.

II. Dilucidar las redes de resistencia cruzada y sensibilidad colateral de poblaciones de *P. aeruginosa* que han evolucionado en presencia de tobramicina, tigeciclina, ceftazidima y ceftazidima-avibactam.

2.3. Estudio de los factores que influyen en la evolución de la resistencia adquirida por mutación en *P. aeruginosa*

I. Evaluar la posible relación epistática existente entre el QS y determinantes de resistencia a antibióticos en *P. aeruginosa*.

II. Estudiar el papel que la presión de selección juega en la evolución de la resistencia de *P. aeruginosa* a tobramicina y tigeciclina.

III. Examinar la robustez de una red de sensibilidad colateral con potencial interés terapéutico en mutantes de *P. aeruginosa* resistentes a distintos antibióticos.

RESULTADOS

3. Resultados

Los resultados de esta tesis están avalados por su publicación en los siguientes artículos científicos:

I. Analysis of the *Pseudomonas aeruginosa* aminoglycoside differential resistomes allows defining genes simultaneously involved in intrinsic antibiotic resistance and virulence.

II. Mutational evolution of *Pseudomonas aeruginosa* resistance to ribosome-targeting antibiotics.

III. Antibiotic resistance evolution is contingent on the quorum-sensing response in *Pseudomonas aeruginosa*.

IV. The evolutionary landscapes of *Pseudomonas aeruginosa* towards ribosome-targeting antibiotics resistance depend on the selection strength.

V. Mutation-driven evolution of *Pseudomonas aeruginosa* in the presence of either ceftazidime or ceftazidime-avibactam.

VI. Rapid and robust evolution of collateral sensitivity in *Pseudomonas aeruginosa* antibiotic-resistant mutants.

Artículo I

Analysis of the *Pseudomonas aeruginosa* aminoglycoside differential resistomes allows defining genes simultaneously involved in intrinsic antibiotic resistance and virulence

Sanz-García, F., Alvarez-Ortega, C., Olivares-Pacheco, J., Blanco, P., Martínez, J. L., Hernando-Amado, S.

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Tradicionalmente, el escrutinio de genotecas de inserción ha sido uno de los métodos de análisis predictivo más fecundos en el estudio del resistoma intrínseco bacteriano. Sin embargo, los trabajos basados en este procedimiento suelen emplear un único antimicrobiano como representante de la familia a la que pertenece, extrapolando los resultados obtenidos a los restantes miembros de ésta. A fin de discernir la validez de esta premisa, que no ha sido lo suficientemente investigada, se analizó la sensibilidad de una compilación de mutantes de inserción de *P. aeruginosa* PA14 (procedentes de un escrutinio previo realizado con amikacina en el laboratorio y dos publicaciones precedentes) a cuatro aminoglicósidos, a saber, tobramicina, amikacina, kanamicina y estreptomicina. Los resultados de este estudio revelaron que la inactivación de cada gen en su correspondiente mutante desembocaba en un fenotipo de sensibilidad diferente para los aminoglicósidos ensayados. Concretamente, sólo dos mutantes de un total de 243 exhibieron cambios semejantes en la sensibilidad a esos cuatro aminoglicósidos, lo que sugiere que el rol de un gen en el resistoma frente a un antibiótico dado no debería ser generalizado al que presentaría frente a los restantes antimicrobianos de su misma familia.

Una vez culminado este aspecto del estudio, se seleccionaron cinco mutantes de inserción que mostraban hipersensibilidad a aminoglicósidos, con el fin de hallar una posible correlación entre este fenotipo y una mayor sensibilidad a otros antibióticos, así como una virulencia exangüe. Los mutantes hipersensibles sometidos a este análisis ulterior fueron aquellos en los cuales el transposón inactivaba los genes *glnD, hflK, PA2798, PA3016* y *hpf*, respectivamente. De entre ellos, el mutante en *glnD* se erigió como el más interesante, puesto que la inactivación de este gen propició hipersensibilidad a varios antibióticos (algunos de reconocida valía clínica, como la fosfomicina y el ciprofloxacino) y una contundente pérdida del potencial virulento de *P. aeruginosa*. La producción de piocianina, formación de biopelículas, actividad elastasa, motilidad de tipo *swarming* y la capacidad de infectar *Caenorhabditis elegans* se vieron significativamente afectadas en este mutante. A fuer de estos resultados, se propone a GlnD como una diana prometedora para el desarrollo de coadyuvantes de antibióticos con posible aplicación en terapias anti-resistencia y anti-virulencia de infecciones debidas a *P. aeruginosa.*

Aportaciones específicas:

Trabajo experimental: Sanz-García, F., Alvarez-Ortega, C., Olivares-Pacheco, J., Blanco, P. y Hernando-Amado, S. contribuyeron a la labor experimental. Concretamente, mi participación se centró en la determinación de la sensibilidad a los 4 aminoglicósidos de los 243 mutantes de inserción, los ensayos de medición de la actividad elastasa, producción de piocianina y capacidad de infectar *C. elegans*; y en la interpretación de los resultados.

Elaboración del manuscrito: todos los autores contribuyeron a la escritura y corrección del manuscrito, llevando a cabo yo la primera versión del mismo.

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MECHANISMS OF RESISTANCE

Analysis of the *Pseudomonas aeruginosa* Aminoglycoside Differential Resistomes Allows Defining Genes Simultaneously Involved in Intrinsic Antibiotic Resistance and Virulence

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ABSTRACT High-throughput screening of transposon insertion libraries is a useful strategy for unveiling bacterial genes whose inactivation results in an altered susceptibility to antibiotics. A potential drawback of these studies is they are usually based on just one model antibiotic for each structural family, under the assumption that the results can be extrapolated to all members of said family. To determine if this simplification is appropriate, we have analyzed the susceptibility of mutants of *Pseudomonas aeruginosa* to four aminoglycosides. Our results indicate that each mutation produces different effects on susceptibility to the tested aminoglycosides, with only two mutants showing similar changes in the susceptibility to all studied aminoglycosides. This indicates that the role of a particular gene in the resistome of a given antibiotic should not be generalized to other members of the same structural family. Five aminoglycoside-hypersusceptible mutants inactivating *glnD*, *hflK*, *PA2798*, *PA3016*, and *hpf* were chosen for further analysis in order to elucidate if lower aminoglycoside susceptibility correlates with cross-hypersusceptibility to other antibiotics and with impaired virulence. Our results indicate that *glnD* inactivation leads to increased cross-susceptibility to different antibiotics. The mutant in this gene is strongly impaired in virulence traits such as pyocyanin production, biofilm formation, elastase activity, and swarming motility and the ability to kill *Caenorhabditis elegans*. Thus, GlnD might be an interesting target for developing antibiotic coadjuvants with antiresistance and antivirulence properties against *P. aeruginosa*.

KEYWORDS intrinsic resistome, *Pseudomonas aeruginosa*, adjuvants, virulence

Pseudomonas aeruginosa is an opportunistic pathogen, widely distributed in nature (1), which causes a variety of nosocomial infections. It is the main cause of chronic infections in patients with cystic fibrosis (CF) or patients afflicted by chronic obstructive pulmonary disease (2, 3). These infections are usually treated with a set of antibiotics that include β-lactams, polymyxins, and aminoglycosides (4), such as tobramycin or amikacin (5, 6). Acquisition of antibiotic-inactivating enzymes through horizontal gene transfer is fundamental in the development of antibiotic resistance by *P. aeruginosa* (7). In addition, resistant mutants are frequently selected during antibiotic treatment, particularly in the case of chronic infections (8–10), which hinder the efficacy of antipseudomonal therapy. Identification of the elements that contribute to a reduced susceptibility to antibiotics, as well as those whose inactivation increases resistance, is relevant for understanding the mechanisms involved in *P. aeruginosa* antibiotic resistance. To this end, high-throughput screening of transposon insertion mutants in search for mutants presenting altered susceptibilities to antimicrobial agents has been shown to be a fruitful strategy (11, 12). Different studies based in the use of transposon-insertion mutants have identified *P. aeruginosa* genes whose inactivation modifies the suscep-

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tibility to antibiotics of this pathogen (13–18). However, these studies are frequently performed using only one antibiotic for each studied group, under the assumption that the results can be extrapolated to other members of the same structural family of antimicrobials (14, 17–19). In the current article, we challenge this hypothesis by analyzing the susceptibility of a large set of *P. aeruginosa* mutants to four aminoglycosides, namely, tobramycin, amikacin, streptomycin, and kanamycin, by using data obtained from in-house screening and previously published data (16, 18). Our studies may help to elucidate whether the use of one antibiotic provides enough information to conclusively assess the implication of a gene in resistance to a whole family of antimicrobials. It is important to highlight that this type of generalization is applied only in the case of mutations in the elements of the intrinsic resistome and not in the case of the acquisition of antibiotic resistance genes. Indeed, it is known that antibiotic-inactivating enzymes (and, in particular, aminoglycoside-inactivating enzymes) are antibiotic specific. We and others have shown that inactivation of the genes that constitute the intrinsic resistome usually produces pleiotropic effects on the susceptibility to antibiotics from different families (17, 20). Consequently, an additional objective of this study was to identify mutants with cross-hypersusceptibility to antibiotics from different structural families. Since inactivation of these genes increases the susceptibility to different antimicrobials, they could be considered suitable targets for the search of antibiotics' coadjuvants.

Taking into consideration that the ever-increasing burden of resistance erodes the efficacy of conventional antibiotics, copious efforts are being made to develop novel therapeutics that block virulence mechanisms, to be used alone or in combination with classical antibiotics (21, 22). The characterization of targets whose inhibition could simultaneously suppress the expression of virulence factors and antibiotic resistance might fuel a field (23) that remains incipient, in spite of recent studies with exactly this focus (24). In this study, we characterized a set of aminoglycoside-hypersusceptible mutants to identify hypothetical connections between hypersusceptibility and impaired virulence. The genes inactivated in these mutants encode potential targets for the development of *P. aeruginosa* antivirulence/antiresistance coadjuvants.

RESULTS

The intrinsic resistome of *P. aeruginosa* to amikacin. An ordered, comprehensive, nonredundant PA14 transposon insertion library (25) was screened to find genes whose inactivation modifies *P. aeruginosa* susceptibility to amikacin. From this screening, 118 mutants displayed changes in their susceptibility to amikacin of at least 2-fold, as determined using an agar dilution method (15, 26). These results included *bona fide* intrinsic resistance genes (genes that contribute to the characteristic phenotype of *P. aeruginosa* susceptibility to amikacin) and genes whose mutation results in low-level amikacin resistance in this pathogen (see Table S1 in the supplemental material).

The shared intrinsic resistome to aminoglycosides of *P. aeruginosa*. Several publications on the intrinsic resistome of bacterial pathogens analyzed only one antibiotic belonging to each structural family in the understanding that the data obtained with one antibiotic might be extrapolated to other members in the family (14, 17–19). To decipher whether or not this was indeed the situation in our case, we included in the analysis genes whose inactivation has been previously reported to modify *P. aeruginosa* susceptibility to aminoglycosides (16, 18). The final number of selected genes was 243 (Table S1). Strains 1 to 118 are the mutants provided from our screening, whereas strains 119 to 122 came from Krahn's work (16) and strains 123 to 243 from Schurek's (18). We chose to use the PAO1 codes of the orthologue counterparts to name the PA14 screened mutants.

The susceptibility of these 243 *P. aeruginosa* PA14 insertion mutants to tobramycin, amikacin, streptomycin, and kanamycin was determined using MIC strips. As shown, 80 transposon-tagged insertion mutants showed changes of at least 3-fold compared to the wild-type strain in their susceptibility to at least one aminoglycoside. Among these, only two mutants showed changes in the MICS of all four studied aminoglycosides (the

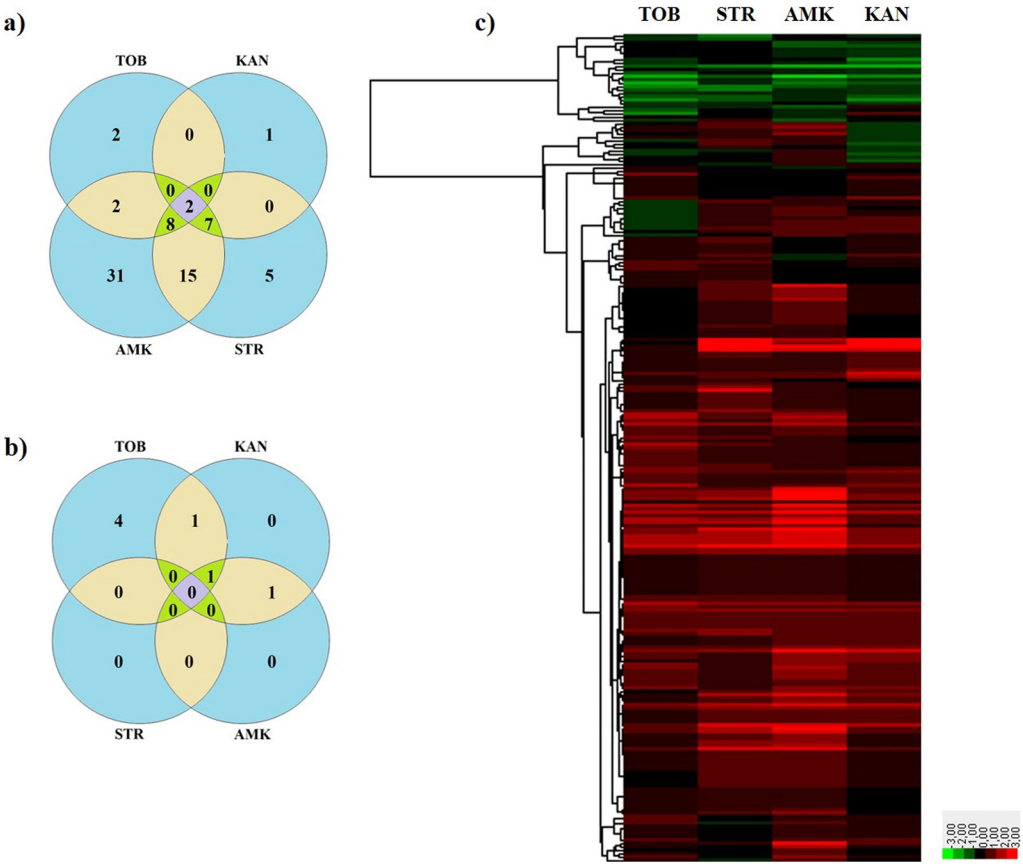


FIG 1 Susceptibility to four aminoglycosides of *P. aeruginosa* mutants. (a and b) Venn diagrams with the number of mutants with changes in their susceptibility to the four aminoglycosides 3-fold above (a) or below (b) the MIC of the parental strain. (c) Hierarchical clustering of the MICs obtained from the aminoglycoside susceptibility screening of 243 *P. aeruginosa* PA14 transposon insertion mutants. The values were represented as $\log_2$ [MIC_{mutant}/MIC_{PA14}], using Gene Cluster 3.0 software and Java Treeview for the graphic display. TOB, tobramycin; KAN, kanamycin. STR, streptomycin; AMK, amikacin. Green represents increased susceptibility and red reduced susceptibility.

ones inactivating *pilC* and *clpS*, the latter being already related to the intrinsic resistome to aminoglycosides of *P. aeruginosa* [27]), 16 in the MICs of three, 19 in the MICs of two, and 43 in the MIC of one (Fig. 1a and b). Notably, these changes were not equally distributed for all aminoglycosides/mutants, and a variety of susceptibility patterns was observed among the studied mutants (Fig. 1c). These results suggest that a change in susceptibility to one antimicrobial agent associated with the inactivation of a given gene does not necessarily imply a similar change in the phenotype of susceptibility to other antibiotics, even when the latter belong to the same structural family.

Cross-susceptibility of *P. aeruginosa* aminoglycoside-hypersusceptible mutants. Among the *P. aeruginosa* PA14 transposon insertion mutants, we selected for further analysis those that exhibited at least a 3-fold increase in susceptibility to at least one aminoglycoside compared to the wild-type strain. The mutants with mutations in genes *PA3658*, *PA4942*, *PA2798*, *PA3016*, and *PA4463* met those requirements. These genes encode GlnD (a protein implicated in N_2 metabolism) (28), HflK (an FtsH protease accessory factor) (29), a probable two-component regulator, a hypothetical protein already described to be involved in intrinsic aminoglycoside resistance (30), and a hibernation-promoting factor (Hpf) that is required for rRNA preservation during starvation (31, 32), respectively (Table S1). Two other mutants, one with a mutation in *mucD* and another with a mutation in *amgS*, were also hypersusceptible but were excluded from further analysis. The *mucD* mutant was excluded because it exhibited an increase in susceptibility to tobramycin and a decrease in susceptibility to kanamycin (Table S1). The role of *amgS* in virulence and resistance to different antibiotics, namely, aminogly-

TABLE 1 MICs of antibiotics of different structural families in the selected *P. aeruginosa* PA14 aminoglycoside-hypersusceptible mutants

Mutant	MIC (μg/ml)[a]								
	TGC	TET	CIP	CAZ	IPM	ATM	FOF	ERY	CHL
PA14	8.0	2.0	0.125	1.0	0.75	2.0	8.0	96.0	24.0
PA3016	6.0	1.5	0.094	1.5	0.75	3.0	6.0	96.0	24.0
hpf	4.0	0.38	0.094	0.5	0.75	2.0	3.0	48.0	24.0
PA2798	3.0	0.5	0.064	1.0	0.75	2.0	1.5	48.0	16.0
hflK	6.0	1.5	0.125	0.75	0.75	1.0	4.0	96.0	24.0
glnD	0.75	0.25	0.064	0.75	0.75	1.5	4.0	64.0	24.0

[a]TGC, tigecycline; TET, tetracycline; CIP, ciprofloxacin; CAZ, ceftazidime; IPM, imipenem; ATM, aztreonam; FOF, fosfomycin; ERY, erythromycin; CHL, chloramphenicol.

cosides, macrolides, quinolones, and β-lactams (30, 33, 34), as well as the nexus between the two-component system it belongs to and the aminoglycoside-promoted expression of the multidrug efflux pump MexXY (35), has already been analyzed in detail and further analysis would thus be redundant. To further confirm the presence of the transposon in these genes, the regions holding it were amplified using specific oligonucleotides (Table S2). To determine if the selected mutants were cross-hypersusceptible to other antibiotics, the MICs of a set of representative antimicrobials were determined. Every mutant, except for the PA3016 mutant, showed higher susceptibility to antibiotics from different structural families (Table 1), implying that the effect of the inactivated genes on antibiotic resistance may not be aminoglycoside specific. All the mutants, except for the PA3016 mutant, presented increased susceptibility to fosfomycin. In addition, all the mutants, except for PA3016 and PA4942, exhibited an increase in susceptibility to tigecycline and tetracycline. The PA3658 mutant displayed the most hypersusceptible phenotype.

***P. aeruginosa* aminoglycoside-hypersusceptible mutants are impaired in their virulence potential.** Besides contributing to *P. aeruginosa* intrinsic antibiotic resistance, the analyzed genes might also impact the production of elements relevant to infection by this bacterial pathogen. To address this possibility, levels of biofilm, elastase, and pyocyanin production, as well as swarming motility phenotypes, were compared between the mutants and the wild-type strain. All the mutants were impaired in biofilm formation, a situation that was especially remarkable in the case of the PA3658, PA2798, and PA4942 mutants (Fig. 2). Additionally, all the mutants exhibited lower elastinolytic activity; however, in this case, the levels of all the mutants were akin (Fig. 2). Concerning pyocyanin synthesis, the level of impairment displayed by the mutants was lower. The PA3016 and PA4463 mutants maintained levels of pyocyanin production similar to those maintained by the parental strain, whereas the mutants with mutations in PA2798, PA3658, and PA4942 showed moderate and yet statistically significant reductions in such production (Fig. 2). Swarming motility was also lessened, with the PA3658 and PA2798 mutants (also largely impaired in biofilm formation and elastinolytic activity) presenting the most anomalous motility patterns (Fig. 3). To determine whether the lower production of virulence factors by the aminoglycoside-hypersusceptible mutants correlates to impaired virulence in an infection model, we performed a *Caenorhabditis elegans* killing assay. As shown in Fig. 4, *P. aeruginosa* PA14 and the PA2798 and PA4463 mutants were the most lethal to *C. elegans*; almost the entire population of worms died within 4 to 5 days. In contrast, the PA3658 mutant appeared to be less lethal, with 9 to 11 nematodes still alive at the end of the experiment. The PA4942 and PA3016 mutants also presented a less lethal phenotype than *P. aeruginosa* PA14, albeit the results were not as clear as the ones obtained with the PA3658 mutant.

DISCUSSION

Within this work, we define new members of the *P. aeruginosa* intrinsic resistome and new potential mechanisms for mutation-driven acquisition of resistance to amin-

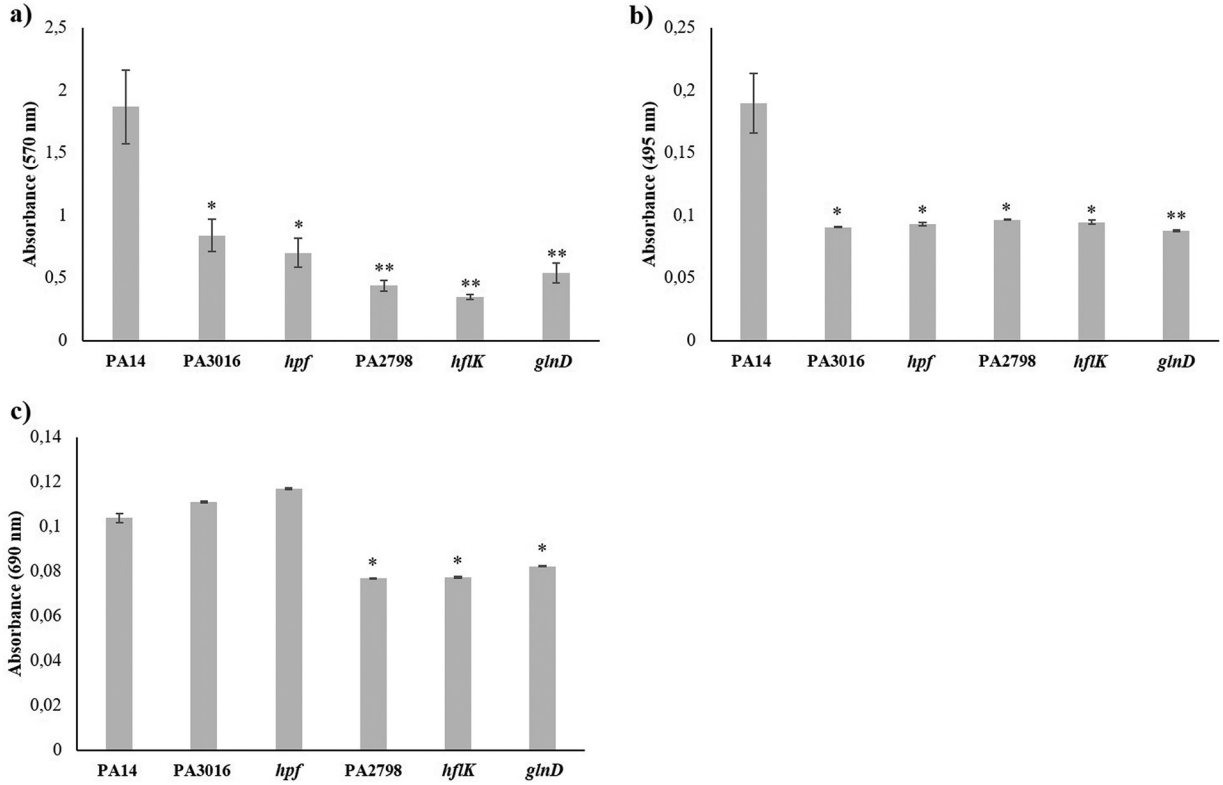
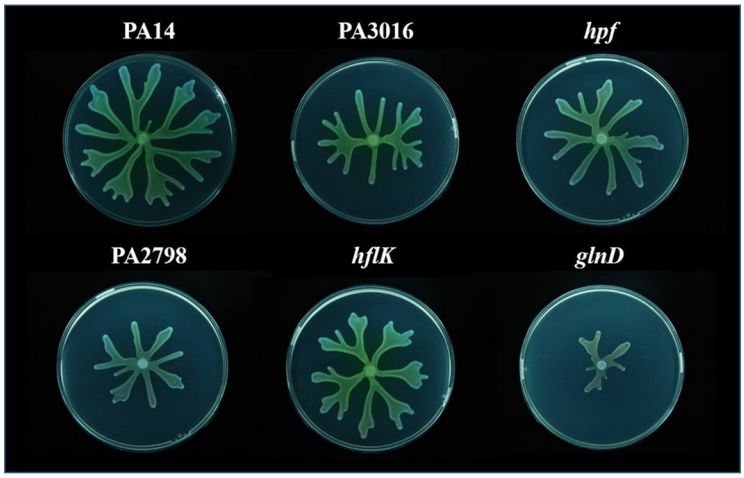


FIG 2 Quantification of different phenotypes with relevance for the virulence of *P. aeruginosa* in aminoglycoside-hypersusceptible mutants. The graphs show (a) biofilm formation assay data, (b) elastase activity data, and (c) pyocyanin production of *P. aeruginosa* PA14 and aminoglycoside-hypersusceptible mutants selected from the screening of the transposon insertion library of this strain. Error bars indicate standard deviations of the results from eight independent experiments in the biofilm formation assay and from three experiments in the other tests. Statistically significant differences with *P* values of <0.05 in the assayed features with respect to the wild-type strain were evaluated using Student's *t* test and are highlighted with one asterisk, whereas *P* values of <0.005 are indicated with two asterisks.

oglycosides. In addition to genes already known to be involved in *P. aeruginosa* intrinsic resistance to aminoglycosides, we identified two novel loci (*glnD* and *mucD*) in the chromosome of *P. aeruginosa* PA14 that contribute to intrinsic resistance to at least one aminoglycoside (see Table S1 in the supplemental material), although inactivation of *mucD* increases susceptibility to one aminoglycoside and reduces susceptibility to another. Interestingly, both genes have been proposed to play a potential role in β-lactam resistance (26, 36). Further, 14 novel loci could potentially be involved in the acquisition of mutational resistance to at least one aminoglycoside, since their inactivation increases the MICs of such aminoglycosides by at least 3-fold compared with the wild-type parental strain. These loci are *PA5183*, *waaL*, *PA1440*, *PA3844*, *PA4874*, *PA1411*, *pilC*, *PA3350*, *nppB*, *flgH*, *flgI*, *nosR*, *ppsA*, and *purF* (Table S1). It is worth mentioning that *pilC* mutant was one of the two mutants that showed a 3-fold MIC increase associated with the four tested aminoglycosides. To date, this gene required for the biogenesis of the *P. aeruginosa* pili has not been reported to be involved in antibiotic resistance. The higher resistance of the mutant could be explained by changes in the membrane potential and permeability due to an anomalous pilus structure, which would affect aminoglycoside uptake.

Note here that mutation-driven resistance is mainly relevant in the case of chronic infections and that resistance to aminoglycosides is frequently due to the acquisition of inactivating enzymes, a feature not analyzed in the current work.

Notably, only a few mutants exhibited simultaneous susceptibility changes with respect to the aminoglycosides included in our study (Fig. 1). This indicates that, while these antibiotics share a mechanism of action, the potential mechanisms of acquiring resistance due to gene inactivation and the elements contributing to intrinsic resistance



Mutants	Diameter (mm)
PA14	77.2
PA3016	51.9
hpf	61.7
PA2798	43.2
hflK	63.3
glnD	30.8

FIG 3 Swarming assay of *P. aeruginosa* PA14 and aminoglycoside-hypersusceptible mutants. The figure shows the swarming of a set of selected mutants in comparison with the PA14 wild-type strain. Three replicates of each mutant were assayed, and pictures were taken after 17 h of incubation at 37°C. The diameters displayed represent means of results from the three replicates.

are not necessarily the same for each of these antibiotics. These results support the idea that, at least in the case of aminoglycosides, the role of a particular gene in the resistome of a given antibiotic cannot be generalized to all members within its family.

The genes whose inactivation increases the susceptibility to antibiotics are suitable

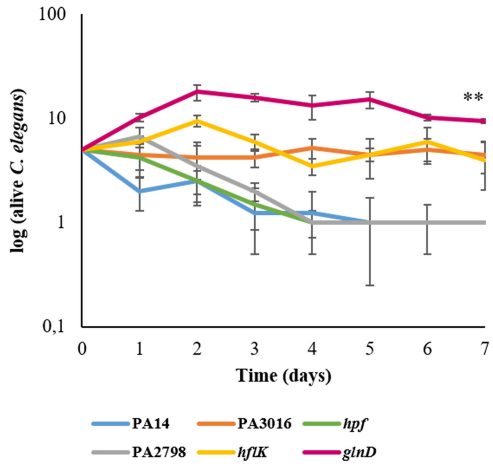


FIG 4 Virulence of *P. aeruginosa* aminoglycoside-hypersusceptible mutants in a *C. elegans* model system. Data present the growth kinetics of *C. elegans* in the presence of either the PA14 wild-type strain or the *P. aeruginosa* hypersusceptible mutants. Error bars indicate standard deviations of the results from four independent experiments. Statistically significant differences ($P < 0.005$) in the survival of nematodes with respect to the wild-type strain were evaluated using Student's *t* test and are highlighted with two asterisks.

targets in the search of coadjuvants able to sensitize bacteria to such elements. Hence, we focused our studies on those mutants that exhibited at least a 3-fold increase in aminoglycosides' susceptibility. In addition to an increase in susceptibility to antibiotics from other structural families, these chosen mutants presented defective phenotypes in biofilm formation, elastase activity, and swarming motility. The PA4463 mutant has increased susceptibility to aminoglycosides and to tetracycline. The mutant is also impaired in biofilm formation and in the production of elastase. *PA4463* (*hpf*) codes for a hibernation-promoting factor that is required for rRNA preservation under prolonged nutrient starvation conditions, including the dormant state of certain subpopulations that are present in *P. aeruginosa* biofilms (31, 32). Interestingly, these biofilms have been shown to tolerate the antibiotics ceftazidime and tobramycin at levels far greater than those necessary to eliminate planktonic bacteria (37, 38). In fact, it is believed that this phenomenon may be due in part to the persistent subpopulations within the biofilms mentioned above, which are able to repopulate them when the treatment finishes (39, 40).

Concerning *hflK* (*PA4942*) and *PA2798*, their role in aminoglycoside resistance has been described previously by Krahn et al. (16) and Hinz et al. (29), respectively. Our results show that, in addition, these mutants are more susceptible to various antimicrobials, in particular, tigecycline, tetracycline, fosfomycin, erythromycin (*PA2798*), and aztreonam and fosfomycin (*hflK*), and are impaired in the production of virulence determinants. The fact that *PA2798* codes for a two-component regulator might be the reason for the effects observed in almost every tested phenotype. Actually, coregulation of biofilm formation, elastase activity, and swimming motility by another common regulator has been recently described in *P. aeruginosa* (41), suggesting these processes to be interconnected. Conversely, HflK is one subunit of the inner membrane protein complex HflKC, which participates in quality control of integral membrane and cytosolic proteins (42). Consequently, one possible cause of the phenotype shown by the mutant lacking this protein may be represented by the pleiotropic effects of the alteration in the complex network of proteases to which HflK belongs, which seems to affect the susceptibility to several classes of antibiotics and to other stressors such as alkaline pH and other compounds (29).

Finally, the PA3658 mutant exhibits increased susceptibility to aminoglycosides, tigecycline, tetracycline, ciprofloxacin, and fosfomycin. In addition, it displays a less lethal action against *C. elegans*, produces less pyocyanin than *P. aeruginosa* PA14, and is strongly impaired in the development of such phenotypes with relevance for infection as biofilm formation, elastase activity, and swarming motility. Thus, inactivation of *glnD* (*PA3658*) results in nonvirulent and nonresistant behavior, although the mechanisms behind this phenotype remain to be established. Note the increased susceptibility to tigecycline and fosfomycin that this mutant showed, because *P. aeruginosa* is intrinsically resistant to tigecycline (43); whereas fosfomycin is one of the antibiotics of choice for the treatment of *P. aeruginosa* infections (4). Very little is known about *glnD* in *P. aeruginosa*, aside from the fact that it encodes an uridylyltransferase associated with the glycine betaine catabolism (44) and its relationship with N_2 catabolism in other bacteria (28). Therefore, further research is needed for understanding the molecular basis of the increased susceptibility to antibiotics and reduced virulence displayed by *P. aeruginosa* when *glnD* is inactivated.

Our results allow the identification of genes that are likely to be simultaneously involved in intrinsic antibiotic resistance and virulence of *P. aeruginosa*. This may unlock new ways of managing and treating infections of this pathogen; for instance, targeting the locus *PA3658* with a proper inhibitor might prevent *P. aeruginosa* from developing a virulent behavior and a resistance phenotype against clinically important antibiotics, such as tobramycin, amikacin, and fosfomycin, or might even dissipate its intrinsic resistance to tigecycline, which would lead us to reconsider the clinical use of this antibiotic upon GlnD inhibition.

MATERIALS AND METHODS

Identification of mutants with altered susceptibility to amikacin. The screening was performed using an agar dilution method as described previously (15, 26). A nonredundant transposon insertion

library of *P. aeruginosa* PA14 (25) harboring 5,850 mutations representing 4,596 genes was used for the screening. PCR amplification was used to verify the presence of the transposon MAR2xT7 in the inactivated genes of five selected hypersusceptible mutants. Five primer pairs, which amplified 150-to-300-bp regions surrounding the transposon in each of the analyzed mutants, were designed (see Table S2 in the supplemental material). After PCR amplification, the sizes of the corresponding amplicons were assessed in comparison to the amplifications in *P. aeruginosa* PA14 strain in a 1% agarose gel.

Analysis of susceptibility to antibiotics. The susceptibility to amikacin, tobramycin, kanamycin, and streptomycin in all selected mutants and to tigecycline, tetracycline, aztreonam, ceftazidime, imipenem, ciprofloxacin, erythromycin, chloramphenicol, and fosfomycin in a subset of strains was determined using MIC strips (MIC Test Strip; Liofilchem) in Mueller-Hinton agar (MHA) (Sigma) at 37°C. The mutants were grouped as a function of their aminoglycoside MICs using Gene Cluster 3.0 software. The hierarchical cluster was displayed using Java Treeview software. MICs were normalized to the value of the wild-type strain using the formula $\log_2$ [MIC_{mutant}/MIC_{PA14}].

Elastase activity and pyocyanin production. The different bacterial strains were cultured at 37°C in 10 ml of LB broth. After 24 h of culture, 1-ml samples were collected and centrifuged for 10 min at 7,000 rpm, and the supernatants were filtered through 0.2-μm-pore-size filters (Whatman). The elastase assay was adapted from a method previously described by Kessler and Safrin (45) as follows: 1 ml of Congo red elastin (Sigma-Aldrich) was added to 100 μl of each sample, and the mixture was incubated at 37°C and 250 rpm for 2 h. Subsequently, samples were centrifuged (10 min, 7,000 rpm) and the optical density at 495 nm (OD_{495}) of 100 μl of the filtered supernatants was determined using a 96-well microtiter plate (Nunc) in a Tecan Infinite M200 plate reader (Tecan). Pyocyanin production was determined by measuring the OD_{690} of 100 μl of filtered supernatants using the same plate reader. Three replicates of each strain were included in the analyses.

Biofilm formation. Biofilm formation was tested using 96-well microtiter plates (Falcon 3911 Microtest III flexible assay plate) previously sterilized with UV light. A modification of a previously reported protocol (46) was followed. A 1:100 dilution of overnight LB broth bacterial cultures was inoculated into the microtiter plate (100 μl/well) and incubated at 37°C for 48 h. Next, 25 μl of a 0.1% crystal violet solution was added to each well (5 min), and the excess dye was repeatedly and thoroughly rinsed with distilled water (4 times). Triton X-100 (0.25%) was added to detach the biofilm from the wells, and 100 μl of each sample was transferred to a 96-well microtiter plate (Nunc). The biofilm quantification was performed by measuring the OD_{570} in a Tecan Infinite M200 plate reader (Tecan). Eight replicates of each strain were included in the assay.

Swarming assay. Swarming assays were performed in petri dishes with 25 ml of a Casamino Acids medium that contained 0.5% Casamino Acids, 0.5% Bacto agar, 0.5% filtered glucose, 3.3 mM K_2HPO_4, and 3 mM $MgSO_4$. A 4-μl inoculum (OD_{600} of 1) of either *P. aeruginosa* PA14 or one of the mutant strains was placed on the center of the agar surface. Three replicates of each strain were incubated for 17 h at 37°C. The diameter of the swarming motility zone was measured and a picture was recorded of every plate.

***Caenorhabditis elegans* virulence assay.** The kinetics of *C. elegans* killing by *P. aeruginosa* PA14 and its derivatives was assessed by using the method previously described by Tan et al. (47), with some modifications. A 50-μl inoculum from each strain (four replicates of each) was grown in 6-cm-diameter plates with potato dextrose agar (PDA; Sigma-Aldrich) for 24 h at 37°C, in order to form a bacterial lawn. Each plate was subsequently seeded with 5 L4-stage hermaphrodite *C. elegans* N2 Bristol worms (48), and plates were incubated at 18°C for a week. Plates were examined for living worms every day during this period. A worm was considered dead when it no longer responded to touch. *E. coli* OP50 was used as a positive control of the preferred food source known to have reduced virulence in *P. aeruginosa*.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00185-19.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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REFERENCES

1. Silby MW, Winstanley C, Godfrey SA, Levy SB, Jackson RW. 2011. Pseudomonas genomes: diverse and adaptable. FEMS Microbiol Rev 35:652–680. https://doi.org/10.1111/j.1574-6976.2011.00269.x.
2. Tummler B, Wiehlmann L, Klockgether J, Cramer N. 2014. Advances in understanding Pseudomonas. F1000Prime Rep 6:9. https://doi.org/10.12703/P6-9.
3. Martinez-Solano L, Macia MD, Fajardo A, Oliver A, Martinez JL. 2008. Chronic *Pseudomonas aeruginosa* infection in chronic obstructive pul-

monary disease. Clin Infect Dis 47:1526–1533. https://doi.org/10.1086/593186.
4. Bassetti M, Vena A, Croxatto A, Righi E, Guery B. 2018. How to manage Pseudomonas aeruginosa infections. Drugs Context 7:212527. https://doi.org/10.7573/dic.212527.
5. Mena A, Smith EE, Burns JL, Speert DP, Moskowitz SM, Perez JL, Oliver A. 2008. Genetic adaptation of *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients is catalyzed by hypermutation. J Bacteriol 190:7910–7917. https://doi.org/10.1128/JB.01147-08.
6. Cantón R, Cobos N, de Gracia J, Baquero F, Honorato J, Gartner S, Alvarez A, Salcedo A, Oliver A, García-Quetglas E. 2005. Antimicrobial therapy for pulmonary pathogenic colonisation and infection by *Pseudomonas aeruginosa* in cystic fibrosis patients. Clin Microbiol Infect 11:690–703. https://doi.org/10.1111/j.1469-0691.2005.01217.x.
7. Holbrook SYL, Garneau-Tsodikova S. 2018. Evaluation of aminoglycoside and carbapenem resistance in a collection of drug-resistant Pseudomonas aeruginosa clinical isolates. Microb Drug Resist 24:1020–1030. https://doi.org/10.1089/mdr.2017.0101.
8. Merlo CA, Boyle MP, Diener-West M, Marshall BC, Goss CH, Lechtzin N. 2007. Incidence and risk factors for multiple antibiotic-resistant Pseudomonas aeruginosa in cystic fibrosis. Chest 132:562–568. https://doi.org/10.1378/chest.06-2888.
9. Burkhardt O, Lehmann C, Madabushi R, Kumar V, Derendorf H, Welte T. 2006. Once-daily tobramycin in cystic fibrosis: better for clinical outcome than thrice-daily tobramycin but more resistance development? J Antimicrob Chemother 58:822–829. https://doi.org/10.1093/jac/dkl328.
10. Geller DE. 2009. Aerosol antibiotics in cystic fibrosis. Respir Care 54:658–670. https://doi.org/10.4187/aarc0537.
11. Gomez MJ, Neyfakh AA. 2006. Genes involved in intrinsic antibiotic resistance of Acinetobacter baylyi. Antimicrob Agents Chemother 50:3562–3567. https://doi.org/10.1128/AAC.00579-06.
12. Martinez JL, Baquero F, Andersson DI. 2007. Predicting antibiotic resistance. Nat Rev Microbiol 5:958–965. https://doi.org/10.1038/nrmicro1796.
13. Alvarez-Ortega C, Wiegand I, Olivares J, Hancock REW, Martínez JL. 2011. The intrinsic resistome of *Pseudomonas aeruginosa* to beta-lactams. Virulence 2:144–146. https://doi.org/10.4161/viru.2.2.15014.
14. Breidenstein EB, Khaira BK, Wiegand I, Overhage J, Hancock RE. 2008. Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. Antimicrob Agents Chemother 52:4486–4491. https://doi.org/10.1128/AAC.00222-08.
15. Fernández L, Alvarez-Ortega C, Wiegand I, Olivares J, Kocíncová D, Lam JS, Martínez JL, Hancock REW. 2013. Characterization of the polymyxin B resistome *of Pseudomonas aeruginosa*. Antimicrob Agents Chemother 57:110–119. https://doi.org/10.1128/AAC.01583-12.
16. Krahn T, Gilmour C, Tilak J, Fraud S, Kerr N, Lau CH, Poole K. 2012. Determinants of intrinsic aminoglycoside resistance in Pseudomonas aeruginosa. Antimicrob Agents Chemother 56:5591–5602. https://doi.org/10.1128/AAC.01446-12.
17. Fajardo A, Martínez-Martín N, Mercadillo M, Galán JC, Ghysels B, Matthijs S, Cornelis P, Wiehlmann L, Tümmler B, Baquero F, Martínez JL. 2008. The neglected intrinsic resistome of bacterial pathogens. PLoS One 3:e1619. https://doi.org/10.1371/journal.pone.0001619.
18. Schurek KN, Marr AK, Taylor PK, Wiegand I, Semenec L, Khaira BK, Hancock RE. 2008. Novel genetic determinants of low-level aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 52:4213–4219. https://doi.org/10.1128/AAC.00507-08.
19. Vestergaard M, Leng B, Haaber J, Bojer MS, Vegge CS, Ingmer H. 2016. Genome-wide identification of antimicrobial intrinsic resistance determinants in Staphylococcus aureus. Front Microbiol 7:2018. https://doi.org/10.3389/fmicb.2016.02018.
20. Liu A, Tran L, Becket E, Lee K, Chinn L, Park E, Tran K, Miller JH. 2010. Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout collection: generating an antibiotic bar code. Antimicrob Agents Chemother 54:1393–1403. https://doi.org/10.1128/AAC.00906-09.
21. Brannon JR, Hadjifrangiskou M. 2016. The arsenal of pathogens and antivirulence therapeutic strategies for disarming them. Drug Des Devel Ther 10:1795–1806. https://doi.org/10.2147/DDDT.S98939.
22. Williams P. 2002. Quorum sensing: an emerging target for antibacterial chemotherapy? Expert Opin Ther Targets 6:257–274. https://doi.org/10.1517/14728222.6.3.257.
23. Martinez JL, Baquero F. 2002. Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance. Clin Microbiol Rev 15:647–679. https://doi.org/10.1128/CMR.15.4.647-679.2002.
24. Guo Q, Wei Y, Xia B, Jin Y, Liu C, Pan X, Shi J, Zhu F, Li J, Qian L, Liu X, Cheng Z, Jin S, Lin J, Wu W. 2016. Identification of a small molecule that simultaneously suppresses virulence and antibiotic resistance of *Pseudomonas aeruginosa*. Sci Rep 6:19141. https://doi.org/10.1038/srep19141.
25. Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, Villanueva J, Wei T, Ausubel FM. 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc Natl Acad Sci U S A 103:2833–2838. https://doi.org/10.1073/pnas.0511100103.
26. Alvarez-Ortega C, Wiegand I, Olivares J, Hancock REW, Martínez JL. 2010. Genetic determinants involved in the susceptibility of *Pseudomonas aeruginosa* to beta-lactam antibiotics. Antimicrob Agents Chemother 54:4159–4167. https://doi.org/10.1128/AAC.00257-10.
27. Sun E, Gill EE, Falsafi R, Yeung A, Liu S, Hancock REW. 27 August 2018. Broad-spectrum adaptive antibiotic resistance associated with Pseudomonas aeruginosa mucin-dependent surfing motility. Antimicrob Agents Chemother https://doi.org/10.1128/AAC.00848-18.
28. Contreras A, Drummond M, Bali A, Blanco G, Garcia E, Bush G, Kennedy C, Merrick M. 1991. The product of the nitrogen fixation regulatory gene nfrX of Azotobacter vinelandii is functionally and structurally homologous to the uridylyltransferase encoded by glnD in enteric bacteria. J Bacteriol 173:7741–7749. https://doi.org/10.1128/jb.173.24.7741-7749.1991.
29. Hinz A, Lee S, Jacoby K, Manoil C. 2011. Membrane proteases and aminoglycoside antibiotic resistance. J Bacteriol 193:4790–4797. https://doi.org/10.1128/JB.05133-11.
30. Lee S, Hinz A, Bauerle E, Angermeyer A, Juhaszova K, Kaneko Y, Singh PK, Manoil C. 2009. Targeting a bacterial stress response to enhance antibiotic action. Proc Natl Acad Sci U S A 106:14570–14575. https://doi.org/10.1073/pnas.0903619106.
31. Akiyama T, Williamson KS, Schaefer R, Pratt S, Chang CB, Franklin MJ. 2017. Resuscitation of Pseudomonas aeruginosa from dormancy requires hibernation promoting factor (PA4463) for ribosome preservation. Proc Natl Acad Sci U S A 114:3204–3209. https://doi.org/10.1073/pnas.1700695114.
32. Williamson KS, Richards LA, Perez-Osorio AC, Pitts B, McInnerney K, Stewart PS, Franklin MJ. 2012. Heterogeneity in Pseudomonas aeruginosa biofilms includes expression of ribosome hibernation factors in the antibiotic-tolerant subpopulation and hypoxia-induced stress response in the metabolically active population. J Bacteriol 194:2062–2073. https://doi.org/10.1128/JB.00022-12.
33. Lau CH, Fraud S, Jones M, Peterson SN, Poole K. 2013. Mutational activation of the AmgRS two-component system in aminoglycoside-resistant Pseudomonas aeruginosa. Antimicrob Agents Chemother 57:2243–2251. https://doi.org/10.1128/AAC.00170-13.
34. Greipel L, Fischer S, Klockgether J, Dorda M, Mielke S, Wiehlmann L, Cramer N, Tummler B. 2016. Molecular epidemiology of mutations in antimicrobial resistance loci of Pseudomonas aeruginosa isolates from airways of cystic fibrosis patients. Antimicrob Agents Chemother 60:6726–6734. https://doi.org/10.1128/AAC.00724-16.
35. Lau CH, Krahn T, Gilmour C, Mullen E, Poole K. 2015. AmgRS-mediated envelope stress-inducible expression of the mexXY multidrug efflux operon of Pseudomonas aeruginosa. Microbiologyopen 4:121–135. https://doi.org/10.1002/mbo3.226.
36. Sanz-Garcia F, Hernando-Amado S, Martinez JL. 24 September 2018. Mutation-driven evolution of Pseudomonas aeruginosa in the presence of either ceftazidime or ceftazidime-avibactam. Antimicrob Agents Chemother https://doi.org/10.1128/AAC.01379-18.
37. Nickel JC, Ruseska I, Wright JB, Costerton JW. 1985. Tobramycin resistance of Pseudomonas aeruginosa cells growing as a biofilm on urinary catheter material. Antimicrob Agents Chemother 27:619–624. https://doi.org/10.1128/AAC.27.4.619.
38. Anwar H, Costerton JW. 1990. Enhanced activity of combination of tobramycin and piperacillin for eradication of sessile biofilm cells of Pseudomonas aeruginosa. Antimicrob Agents Chemother 34:1666–1671. https://doi.org/10.1128/AAC.34.9.1666.
39. Mulcahy LR, Burns JL, Lory S, Lewis K. 2010. Emergence of Pseudomonas aeruginosa strains producing high levels of persister cells in patients with cystic fibrosis. J Bacteriol 192:6191–6199. https://doi.org/10.1128/JB.01651-09.
40. Lewis K. 2007. Persister cells, dormancy and infectious disease. Nat Rev Microbiol 5:48–56. https://doi.org/10.1038/nrmicro1557.
41. Li Y, Bai F, Xia H, Zhuang L, Xu H, Jin Y, Zhang X, Bai Y, Qiao M. 2015. A novel regulator PA5022 (aefA) is involved in swimming motility, biofilm

formation and elastase activity of Pseudomonas aeruginosa. Microbiol Res 176:14–20. https://doi.org/10.1016/j.micres.2015.04.001.
42. Kihara A, Akiyama Y, Ito K. 1996. A protease complex in the Escherichia coli plasma membrane: HflKC (HflA) forms a complex with FtsH (HflB), regulating its proteolytic activity against SecY. EMBO J 15:6122–6131. https://doi.org/10.1002/j.1460-2075.1996.tb01000.x.
43. Martinez JL, Coque TM, Baquero F. 2015. What is a resistance gene? Ranking risk in resistomes. Nat Rev Microbiol 13:116–123. https://doi.org/10.1038/nrmicro3399.
44. Wargo MJ, Szwergold BS, Hogan DA. 2008. Identification of two gene clusters and a transcriptional regulator required for Pseudomonas aeruginosa glycine betaine catabolism. J Bacteriol 190:2690–2699. https://doi.org/10.1128/JB.01393-07.
45. Kessler E, Safrin M. 2014. Elastinolytic and proteolytic enzymes. Methods Mol Biol 1149:135–169. https://doi.org/10.1007/978-1-4939-0473-0_13.
46. O'Toole GA, Kolter R. 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol 28:449–461. https://doi.org/10.1046/j.1365-2958.1998.00797.x.
47. Tan MW, Rahme LG, Sternberg JA, Tompkins RG, Ausubel FM. 1999. *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. Proc Natl Acad Sci U S A 96:2408–2413.
48. Brenner S. 1974. The genetics of Caenorhabditis elegans. Genetics 77:71–94.

Artículo II

Mutational evolution of *Pseudomonas aeruginosa* resistance to ribosome-targeting antibiotics

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Este trabajo describe los resultados obtenidos de una ALE de *P. aeruginosa* PA14 en presencia de dos antibióticos que comparten el ribosoma como diana: tobramicina y tigeciclina. Si bien el primero forma parte de la terapia al uso contra este microorganismo, *P. aeruginosa* es intrínsecamente resistente a la tigeciclina. Empero, esto no es óbice para que este patógeno pueda dar lugar a superinfecciones en pacientes que han sido tratados con tigeciclina, cobrando por tanto relevancia el efecto que ésta pueda ejercer sobre su fenotipo de sensibilidad a otros antibióticos.

La evolución experimental fue realizada con concentraciones crecientes de los dos antimicrobianos (llegando hasta 32 veces la CMI de la cepa parental) y culminada con el análisis de las mutaciones presumiblemente responsables de la resistencia alcanzada y el seguimiento de su orden de aparición. De esta disquisición se destiló el papel prominente en la resistencia a tobramicina de mutaciones en genes como *fusA* y *ptsP*, y de la bomba de expulsión MexCD-OprJ, en lo que a la tigeciclina atañía. Asimismo, un aspecto interesante de este estudio residió en el considerable grado de conservación del que hicieron gala las trayectorias evolutivas de las réplicas de *P. aeruginosa* hacia la resistencia a cada uno de estos antibióticos -especialmente tobramicina-, con incluso mutaciones en algún gen en común seleccionadas bajo ambas presiones selectivas (*orfN* y *pmrB*). Así, estos resultados refuerzan la idea de que la evolución de la resistencia con base en la selección de mutaciones dista de ser totalmente estocástica, ya que en este trabajo se observa un razonable nivel de determinismo, que insufla nuevas esperanzas a la prognosis de la resistencia a estos antimicrobianos.

Por último, a nivel fenotípico cabe destacar el notable incremento en la resistencia a tigeciclina que mostraron las poblaciones que evolucionaron en su presencia. Tal hecho demuestra que una bacteria puede evolucionar hacia niveles mayores de resistencia frente a un antibiótico, aun siendo considerada intrínsecamente resistente al mismo. Además, la aplicación de tigeciclina generó resistencia cruzada frente a antibióticos de interés clínico, como ciprofloxacino, ceftazidima o distintos aminoglicósidos; situación alarmante que debería ser tenida en cuenta a la hora de escoger la terapia más conveniente para superinfecciones que puedan contener *P. aeruginosa* en pacientes anteriormente tratados con tigeciclina.

Aportaciones específicas:

Trabajo experimental: Sanz-García, F. y Hernando-Amado, S. contribuyeron a la labor experimental. Concretamente, mi participación se centró en el análisis bioinformático de los genomas secuenciados de las poblaciones evolucionadas, la confirmación de la existencia de las mutaciones identificadas y determinación de su orden de aparición, la cuantificación de la sensibilidad a antibióticos distintos al de selección en las poblaciones mentadas, la medición del nivel de expresión de *mexCD-oprJ* y la interpretación de los resultados.

Elaboración del manuscrito: todos los autores contribuyeron a la escritura y corrección del manuscrito, llevando a cabo yo la primera versión del mismo.

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Mutational Evolution of *Pseudomonas aeruginosa* Resistance to Ribosome-Targeting Antibiotics

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The present work examines the evolutionary trajectories of replicate *Pseudomonas aeruginosa* cultures in presence of the ribosome-targeting antibiotics tobramycin and tigecycline. It is known that large number of mutations across different genes – and therefore a large number of potential pathways – may be involved in resistance to any single antibiotic. Thus, evolution toward resistance might, to a large degree, rely on stochasticity, which might preclude the use of predictive strategies for fighting antibiotic resistance. However, the present results show that *P. aeruginosa* populations evolving in parallel in the presence of antibiotics (either tobramycin or tigecycline) follow a set of trajectories that present common elements. In addition, the pattern of resistance mutations involved include common elements for these two ribosome-targeting antimicrobials. This indicates that mutational evolution toward resistance (and perhaps other properties) is to a certain degree deterministic and, consequently, predictable. These findings are of interest, not just for *P. aeruginosa*, but in understanding the general rules involved in the evolution of antibiotic resistance also. In addition, the results indicate that bacteria can evolve toward higher levels of resistance to antibiotics against which they are considered to be intrinsically resistant, as tigecycline in the case of *P. aeruginosa* and that this may confer cross-resistance to other antibiotics of therapeutic value. Our results are particularly relevant in the case of patients under empiric treatment with tigecycline, which frequently suffer *P. aeruginosa* superinfections.

Keywords: antibiotic resistance, *Pseudomonas aeruginosa*, tobramycin, tigecycline, mutation, evolution

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INTRODUCTION

Antibiotic resistance has been a major public health concern since the dawn of the antibiotic era, but in recent decades there has been an alarming increase in the number and type of antibiotic-resistant bacteria (Appelbaum, 2012), posing a threat to health worldwide (Roca et al., 2015). Predicting the mechanisms by which bacteria may acquire resistance is therefore important in the prevention and treatment of infections (Martinez et al., 2007; Martinez et al., 2011).

Resistance can be acquired via horizontal transfer of antibiotic resistance genes (HGT), or through mutation (Hernando-Amado et al., 2017). While exhaustive information is available on the mechanisms of antibiotic resistance at the basic science and epidemiological levels, the evolutionary trajectories leading to high level antimicrobial resistance, as well as the reproducibility of these trajectories among populations evolving concurrently, have been studied in less detail. It is worth mentioning, however, that the reconstruction of mutants that are selected in patients

under treatment have shown that fitness costs and the selection of compensatory mutations are critical for the success of some specific antibiotic resistance mutations (Shcherbakov et al., 2010; Brandis et al., 2015; Meftahi et al., 2016; Huseby et al., 2017). Nevertheless, this type of retrospective analyses is useful just for studying already known mechanisms of resistance, not for predicting new ones (Pietsch et al., 2017).

Strategies to predict the emergence of resistance mutations (Martinez et al., 2007) were implemented soon after the discovery of antibiotics (Bryson and Szybalski, 1952), one of the most useful of which is experimental evolution. Since the seminal work of Richard Lenski, experimental evolution has been used to analyze different bacterial traits, including the development of resistance to antibiotics (Bryson and Szybalski, 1952; Toprak et al., 2011; Turrientes et al., 2013; Feng et al., 2016; Ibacache-Quiroga et al., 2018). Recent research has shown experimental evolution able to predict the emergence of resistance against different antimicrobial agents, including colistin (Jochumsen et al., 2016), beta-lactams, quinolones and aminoglycosides (Cabot et al., 2016; Feng et al., 2016; Ibacache-Quiroga et al., 2018; Lopez-Causape et al., 2018b). In recent years, the potential of experimental evolution has been further boosted by the development of technologies that allow the fast and affordable sequencing of whole bacterial genomes.

Pseudomonas aeruginosa, an opportunistic pathogen widely distributed in nature (Silby et al., 2011), commonly causes lung, airway and other infections in hospitalized patients. It is the main cause of chronic infections in patients with cystic fibrosis (CF) and chronic obstructive pulmonary disease (Martinez-Solano et al., 2008; Tummler et al., 2014). These infections are usually fought using aminoglycosides, β-lactams and polymyxins (Palmer and Whiteley, 2015). Unfortunately, *P. aeruginosa* intrinsically shows low-level susceptibility to a number of drugs, even against the recently developed glycylcycline tigecycline (Pankey, 2005), which works via tightly binding to the ribosome and thus evading the most common tetracycline resistance mechanisms. In addition, mutants presenting increased levels of antibiotic resistance are selected along chronic infections. Indeed, while resistance to aminoglycosides in *P. aeruginosa* isolates from acute infections has been largely attributed to the acquisition of antibiotic resistance genes, mutation plays a major role for the acquisition of resistance by *P. aeruginosa* causing chronic infections (Vogne et al., 2004; Guenard et al., 2014; Bolard et al., 2018).

In the present work, experimental evolution and whole-genome sequencing (WGS) were used to examine evolutionary trajectories of *P. aeruginosa* toward resistance against two ribosome-targeting antimicrobials: tobramycin and tigecycline. The aim was to determine whether the mechanisms that impair the actions of different drugs targeting the same cell machinery are shared (at least in part), or whether resistance to each drug is specific via a different mechanism. Tigecycline binds to the 30S ribosomal subunit, thereby blocking the interaction of aminoacyl-tRNA with the A site of the ribosome, whereas tobramycin prevents the formation of the 70S complex (Kotra et al., 2000). While tobramycin forms part of usual therapy regimens against *P. aeruginosa* (Cheer et al., 2003), the pathogen is intrinsically resistant to tigecycline (following the clinical definition of antibiotic resistance) (Martinez et al., 2015). One cause of this phenotype is the capability of the multidrug efflux pump MexXY, also involved in intrinsic *P. aeruginosa* tobramycin resistance (Westbrock-Wadman et al., 1999), to extrude tigecycline (Dean et al., 2003). Nonetheless, tigecycline was used in the present study since it provides an opportunity to examine whether or not bacterial pathogens can acquire clinically relevant characteristics when challenged with the antibiotics to which they are considered to be intrinsically resistant. In this regard, it is worth mentioning that *P. aeruginosa* has emerged as a major cause of superinfection in nosocomial patients treated with tigecycline (Garcia-Cabrera et al., 2010; Ulu-Kilic et al., 2015; Katsiari et al., 2016). Knowing whether or not the empirical use of tigecycline for treating Gram-negative hospital infections might challenge *P. aeruginosa*, affecting its susceptibility to other antibiotics commonly used for treating *P. aeruginosa* infections, is of relevance for developing a rational approach for treating such superinfections. The results showed that even for microorganisms dubbed intrinsically resistant to an antibiotic, the challenge with this antibiotic selects mutants presenting reduced susceptibility to antibiotics of clinical value.

Experimental evolution studies allow one to determine whether evolutionary trajectories are reproducible, i.e., whether the process of evolution is mainly deterministic and hence predictable (Martinez et al., 2007), or whether it is largely stochastic. The present work provides a predictive analysis of the potential mutational causes of resistance in *P. aeruginosa* against two ribosome-targeting antibiotics belonging to different structural families, as well as the different evolutionary trajectories taken toward this resistance. This information may allow new strategies to be designed for predicting, managing, and eventually reducing resistance in this important nosocomial pathogen. In addition, the results throw light on whether bacterial evolution is largely stochastic or presents some deterministic features, and thus whether the emergence and spread of antibiotic resistance can be predicted to a certain extent (Martinez et al., 2007, 2011).

MATERIALS AND METHODS

Growth Conditions and Determination of Susceptibility to Antibiotics

Unless otherwise stated, bacteria were grown in Mueller Hinton Broth (MHB, Pronadisa) at 37°C with agitation at 250 rpm. The initial concentrations of tigecycline (Pfizer) and tobramycin (Normon, S. L) that inhibit the growth of *P. aeruginosa* PA14 under the culture conditions used in the evolution experiments were determined at 37°C.

General susceptibility to a wide range of antibiotics - tigecycline, tetracycline, aztreonam, ceftazidime, imipenem, ciprofloxacin, levofloxacin, norfloxacin, tobramycin, streptomycin, amikacin, colistin, polymyxin B, chloramphenicol, fosfomycin and erythromycin – was examined by disk diffusion in Mueller Hinton Agar (MHA) (Sigma) at 37°C.

The MICs of different antibiotics were determined for the bacterial populations over the evolution period at 37°C in MHA using *E*-test strips (MIC Test Strip, Liofilchem®). MICs of colistin and polymyxin B were determined in MHB II by double dilution in microtiter plates. The MICs to the antibiotics of selection and to fosfomycin were repeated twice and in all cases, the results were the same in the replicated assays.

Experimental Evolution

Twelve independent bacterial populations (four controls without antibiotics, four populations challenged with tigecycline, and four populations challenged with tobramycin) were grown in parallel in MHB for 35 consecutive days. All replicates were established from a stock culture of the *P. aeruginosa* PA14 strain. Each day, the cultures were diluted (1/250) with fresh MHB: 8 μl of bacterial culture in 2 ml of medium. The concentrations of tigecycline and tobramycin used for selection increased over the evolution period from the initial MIC up to 32MIC, doubling them every 5 days. Every 5 days, samples from each culture were taken and preserved at −80°C for future investigation.

Whole-Genome Sequencing

Genomic DNA was extracted at the end of the evolution assays from all 12 populations using the Gnome® DNA kit (MP Biomedicals). Whole-genome sequencing was performed by Sistemas Genómicos S.L. Libraries were obtained without amplification following Illumina protocols and recommendations. The quality of the extracted material was analyzed via a 4200 Tapestation High Sensitivity assay, and the DNA concentration determined by real-time PCR using a LightCycler 480 device (Roche). The pool of libraries was pair-end sequenced (100 × 2) in an Illumina HiSeq 2500 sequencer. The average number of reads per sample was 8646177, which represents a coverage of 200x on average. Short reads used in this publication are deposited in SRA database[1] with accession PRJNA490803.

Bioinformatic Analysis

Mutations in the evolved bacteria were detected using CLC Genomics Workbench 9.0 (QIAGEN) software. WGS data were trimmed and the reads aligned with the *P. aeruginosa* UCBPP-PA14 reference chromosome (NC_008463.1). The single nucleotide polymorphisms (SNPs) present in the populations kept under selective pressure were identified and filtered against those present in the populations maintained in the absence of such pressure. The cut-off threshold of a mutation to be included in the analysis was ≥15%.

Confirmation of SNPs

Sanger sequencing was used to verify the mutations found via WGS (**Supplementary Table S1**) and to ascertain the order of appearance of these modifications. Twenty-four pairs of primers, which amplified 100–200 base pair regions containing each putative mutation, were designed (**Supplementary Table S2**). After PCR amplification, the corresponding amplicons were purified using the QIAquick PCR Purification Kit (QIAGEN) and sequenced at GATC Biotech.

[1]https://www.ncbi.nlm.nih.gov/sra

RNA Extraction and Real-Time RT-PCR

One flask containing 20 ml of MHB was inoculated with an overnight culture of the selected clones and *P. aeruginosa* PA14 to a final $O.D._{600}$ = 0.01, and they were incubated until exponential phase was reached ($O.D._{600}$ = 0.6). 10 ml of each culture were centrifuged at 7000 rpm for 15 min and at 4°C. This process was performed with three independent biological replicates.

Then, RNeasy mini Kit (QIAGEN) extraction protocol was followed: 570 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and 30 μl of lysozyme (Sigma), for a final concentration of 1 mg/ml of the latter, were added to each sample. Afterward, the samples were mixed by vortexing for 10 s and were incubated at room temperature for 10 min with regular vortexing. A volume of 2100 μl of buffer RLT (QIAGEN) was added, and samples were sonicated at 0.45 Hz for 20 s. Next, 1410 μl of ethanol (Merck) was added and the protocol continued according to the manufacturer's instructions. In order to remove any residual DNA, two DNase treatments were carried out, with DNase I (QIAGEN) and TURBO DNase (Ambion). Finally, a PCR with *rplU* primers (**Supplementary Table S2**) was performed to check that no residual DNA was present in the RNA samples. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to synthesize cDNA from 5 μg of RNA. Then, real-time RT-PCR was performed using 50 ng of cDNA and the Power SYBR green PCR Master Mix (Applied Biosystems) in the ABI PRISM 7500 real-time PCR system (Applied Biosystems). Gene expression data were normalized using the gene *rplU* (**Supplementary Table S2**). Differences in the relative amounts of mRNA were obtained following the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

Different publications have shown that the genes that contribute to antibiotic resistance (intrinsic resistome) comprise around 3% of the genome of a given bacterial species (Breidenstein et al., 2008; Fajardo et al., 2008; Tamae et al., 2008; Alvarez-Ortega et al., 2010; Blake and O'Neill, 2013; Fernandez et al., 2013). In the case of *P. aeruginosa* the search of a comprehensive transposon-tagged library has shown that the inactivation of 135 genes renders low-level tobramycin resistance (Schurek et al., 2008). Even if we do not take into consideration gain of function mutations (which are not considered in transposon insertion libraries studies), neither that different mutations might be selectable in each of these genes (Martinez and Baquero, 2000), hence increasing the number of potential antibiotic resistance mutants (Lopez-Causape et al., 2018a), the number of possible combinations of these 135 genes is 2.7E230. It can be argued that the number of mutations that a single bacterium can accumulate is likely low. However, even if only five mutations are accumulated as reported in the present work (see below), the number of combinations of these 135 potential mutations, taken five by five, is 4.1E10 if the order of

selection is taken into consideration and 3.4E8 if the order of selection of each of the mutations along evolution is not taken into consideration. If all possible combinations were equivalent in terms of antibiotic resistance, these numbers would mean that mutation-driven antibiotic resistance should be un-predictable.

Stepwise Evolution of *P. aeruginosa* Toward Antibiotic Resistance

To determine whether similar potential evolutionary trajectories are followed by different populations, four biological replicates were allowed to evolve in parallel under selective pressure from tobramycin (populations 1–4), tigecycline (populations 5–8) and in the absence of any selective pressure (populations 9–12). In populations 1–8 the antibiotic concentration was doubled every 5 days, from 0.5 μg/ml for tobramycin and 4 μg/ml for tigecycline, up to 32MIC. At 64MIC no growth was seen after 5 days incubation, suggesting this concentration to be beyond limits of *P. aeruginosa* when evolving toward tobramycin and tigecycline resistance in these experimental conditions.

To monitor the evolution of resistance over the selection period, the susceptibility of each population to the selecting antibiotic was determined every 5 days by *E*-test. When bacteria are confronted to increased concentrations of antibiotics, two different phenotypic trajectories can be foreseen. Sudden selection of high-level resistance at the first steps of evolution or stepwise selection of mutants presenting increasing resistance levels. As shown in **Figure 1**, a stepwise evolutionary trajectory was observed for both antibiotics, suggesting either accumulation of sequential mutations after each evolution step (i.e., each change in antibiotic concentration), or the displacement of low-level resistance mutants by higher-level resistance ones as the selection pressure increased. It is important to note that the evolutionary trajectory was similar in all replicated experiments (**Figure 1**). This suggests the number of evolutionary trajectories possible (at least at phenotypic level) is limited, although the amount of genotypic evolutionary trajectories can be larger (Lassig et al., 2017).

An increase in the MIC of an antibiotic after experimental evolution does not, however, necessarily mean that antibiotic-resistant mutants have been selected for: resistance may be due to a phenotypic adaptation to the presence of an antibiotic rather than to mutations (Levin and Rozen, 2006; Martinez et al., 2009; Martinez and Rojo, 2011). To address this possibility, the evolved populations were sub-cultured in the absence of selection pressure (three sequential passages in MHB) and the MICs again determined. These were found not to change, indicating that the observed modifications were mainly due to the selection of stable mutants.

Cross-Resistance and Collateral Sensitivity of Evolved Populations

To determine whether the development of resistance was specific to the selecting antibiotic or also affected susceptibility to other antimicrobials, a range of representative antibiotics was tested (beta-lactams, quinolones, tetracyclines, macrolides, aminoglycosides, polymyxins, and chloramphenicol) by disk diffusion (**Supplementary Table S4**). Despite it has been shown macrolides as azithromycin can select *mexCD-oprJ* overpressing mutants in *P. aeruginosa* biofilms (Mulet et al., 2009), no differences in susceptibility to imipenem and erythromycin were detected between the wild-type parental *P. aeruginosa* PA14 strain and the evolved populations. Whether these differences can be due to the different experimental model (biofilm or planktonic cells) or the different genetic background where mutants are selected (PAO1 or PA14) remains to be established. Nevertheless, almost every evolved population developed resistance against other antibiotics belonging to the different structural families, implying that at least some resistance mutations are not tigecycline- or tobramycin-specific (**Table 1**). Notably, in all cases the evolved populations were more susceptible to fosfomycin than the wild-type strain, and it is important to know whether this is related to the development of resistance to ribosome-targeting agents. In this regard, it is worth mentioning that *Listeria monocytogenes* is more susceptible to fosfomycin when growing intracellularly than when growing extracellularly. The reason for this lies in the overexpression of a hexose-phosphate transporter when the bacteria are grown intracellularly, which provides an entry route for this antibiotic (Scortti et al., 2006). These results indicate that the resistance to ribosome-targeting drugs may correlate with increasing susceptibility to fosfomycin, although the underlying mechanisms remain obscure. Given that these types of drug are widely used clinically, it is important to determine whether this trade-off occurs commonly, and whether the combined or sequential use of both types of antibiotic offers a better alternative to current therapies.

Mutations Selected in the Presence of Antibiotics

To gain insight into the genetic events associated with the development of resistance in the evolved populations, the genomes of each, as well as that of the original population (for which a frozen sample was available), were sequenced on the last day of the experiment (when the antibiotic concentration was 32MIC). Different mutations can appear by chance or because of the *P. aeruginosa* adaptation for growing in MHB. Consequently, only those mutations present in the populations evolving under antibiotic selective pressure and not in the control populations evolving in absence of selection were taken into consideration. In other words, only those mutations that are enriched (and hence have been selected) under antibiotic selective pressure were taken into consideration. Indeed, all mutant alleles selected in the presence of antibiotics and not present in the populations grown without antibiotics present always a coverage >50% and typically >90% (**Supplementary Table S1**) in the whole population, indicating that they are under positive selection when *P. aeruginosa* grows in presence of antibiotics. **Supplementary Table S1** shows the locations of all 35 confirmed genetic changes potentially associated with the development of resistance. A total of 31 single-nucleotide variants (SNVs) and 4 multi-nucleotide variants (MNVs; deletions and substitutions of various nucleotides and one transposition) were found, 31 located in genes and 4 in intergenic regions. The majority of the

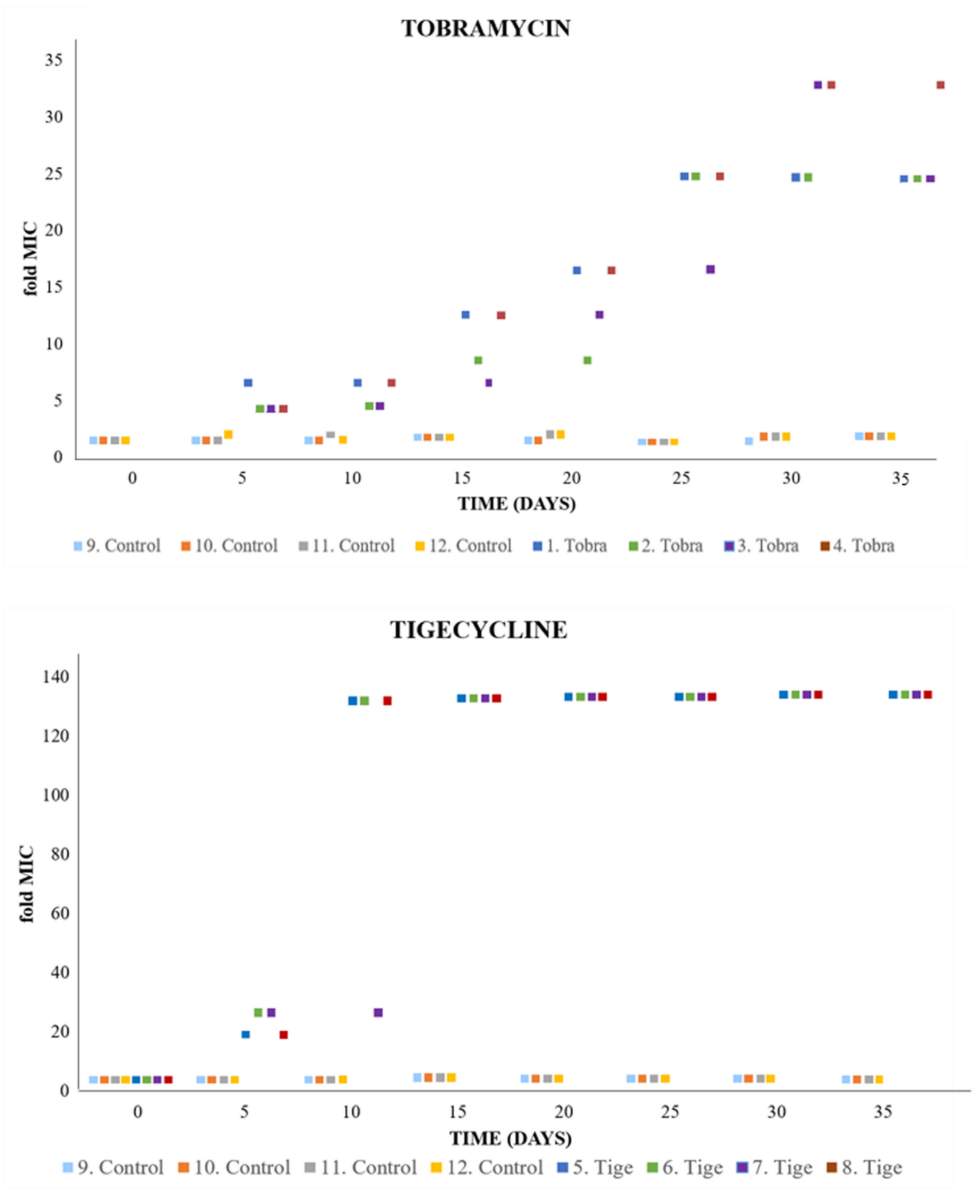

FIGURE 1 | Evolution of *P. aeruginosa* under antibiotic selective pressure. Graphs show the rise of the MICs over the evolution period, from the value corresponding to the wild-type strain (tobramycin [Tobra], 1 μg/ml; tigecycline [Tige], 2 μg/ml) to high levels of tobramycin/tigecycline resistance (doubling the antibiotic concentration every 5 days). The detection limit of the tigecycline *E*-test was 256 μg/ml, limiting the assessment of resistance levels from day 15 to the end of the experiment. MIC values for each replicate are provided in **Supplementary Table S3**.

mutations located in genes resulted in amino acid alterations, frameshifts or stop codons. In addition, the coverage of the obtained reads was mapped to the *P. aeruginosa* genome to search for gene amplifications and deletions. One 377 bp deletion was detected in the tigecycline-treated population 8, comprising part of the gene coding for the transcriptional repressor of the multidrug efflux pump *mexCD-oprJ*, *nfxB*, plus a small region of the adjacent gene *morA*.

To further verify the presence in the evolved populations of the mutations identified by WGS (**Supplementary Table S1**), the regions containing these mutations were amplified and the amplicons Sanger-sequenced.

Common Aspects of the Evolution of *P. aeruginosa* Toward Resistance to Tobramycin or Tigecycline

The present results shed light on the development of resistance to ribosome-targeting antibiotics in *P. aeruginosa*. Although this opportunistic pathogen is already intrinsically resistant to

TABLE 1 | MICs (μg/ml) of antibiotics of different structural families in the populations evolved at 32MIC tobramycin and tigecycline.

Replicate	Tgc	Tet	Atm	Caz	Cip	Tob	S	Ak	C	F	Cs	PB
PA14	1.5	16	1.5	1	0.094	1	16	2	24	24	1.5	0.75
					Tobramycin 32MIC							
1	64	48	3	1.5	0.5	32	192	≥256	24	1	6	2.5
2	24	32	4	1.5	0.5	24	256	192	32	1	6	2.5
3	12	24	4	1.5	0.19	8	64	128	24	1	4	3
4	32	24	3	1.5	0.5	32	192	≥ 256	24	1.5	5	2.5
					Tigecycline 32MIC							
5	≥256	192	8	4	0.75	4	96	32	128	2	2.5	1
6	≥256	192	8	3	0.5	1.5	64	16	64	1.5	2.5	1
7	≥256	≥256	6	3	0.25	2	128	16	96	1	5	2
8	≥256	192	6	2	0.75	8	192	32	128	2	5	1.5
					Controls							
9	2	12	2	1.5	0.094	1	12	4	24	32	1.5	0.75
10	2	8	2	1	0.19	1	12	2	16	32	1.5	0.75
11	1.5	12	2	1	0.094	1	12	3	32	32	1.5	1
12	2	12	2	1.5	0.125	0.75	12	3	16	32	1.5	0.75

Tgc, tigecycline; tet, tetracycline; atm, aztreonam; caz, ceftazidime; cip, ciprofloxacin; tob, tobramycin; s, streptomycin; ak, amikacin; c, chloramphenicol; f, fosfomycin; cs, colistin; pb, polymyxin B. All MICs were obtained by E-test, excepting for polymyxin B and colistin, which were analyzed via double dilution in a microtiter plate.

TABLE 2 | Previously described role on antibiotic resistance of genes presenting mutations in the evolved populations.

Gene name	Tobramycin replicates	Tigecycline replicates	Previously described role in resistance	References
orfN	4	4	Ciprofloxacin	Wong et al., 2012
pmrB	4	2	Cationic peptides, quinolones, tigecycline, tobramycin	Muller et al., 2011; Moskowitz et al., 2012; Lopez-Causape et al., 2018b
fusA	4	–	Aminoglycosides	Wang et al., 2015; Feng et al., 2016; Bolard et al., 2018; Lopez-Causape et al., 2018b
ptsP	2	–	Tobramycin	Schurek et al., 2008
fleQ	1	–	–	–
nfxB	–	4	Quinolones, tetracyclines, β-lactams, chloramphenicol	Masuda et al., 2000b
mexCD	–	2; 1 *mexC*, 1 *mexD*	Quinolones, tetracyclines, β-lactams, chloramphenicol	Masuda et al., 2000b
PA14_00180	–	2	–	–
rpsj	–	2	Tetracycline, tigecycline	Hu et al., 2005; Ahn et al., 2016
parRS	–	3; 2 *parR*, 1 *parS*	Cationic peptides, aminoglycosides	Fernández et al., 2010; Muller et al., 2011
secAG	–	2; 1 *secA*, 1 *secG*	–	–
frr	–	1	–	–
rpoN	–	1	Carbenicillin, quinolones, tobramycin	Viducic et al., 2007, 2016, 2017

tigecycline, highly resistant populations with MICs several times higher than that seen for the wild-type strain were selected for in the experimental evolution assays (**Figure 1**).

Overall, the results indicate that increased resistance to tigecycline and tobramycin comes about via distinguishable evolutionary trajectories but which show some similarities (**Table 2**). In particular, mutations in *orfN* (selected in all eight replicates) and *pmrB* (selected in 6 out of 8 replicates) were selected under pressure from either antibiotic (**Figure 2**). It should be noted that all populations evolved in the presence of antibiotic, and none of those evolved in unexposed control populations show mutants in *orfN* during the first step in evolution (i.e., after the first change in antibiotic concentration). This gene codes for a putative glycosyl transferase needed for the glycosylation of type A flagellins (Schirm et al., 2004). Six of the studied mutants carried a single base pair deletion in a poly-G repeat in *orfN*, and two contained a single base pair insertion in the same region (**Supplementary Table S1**), all of them leading to a Val50fs mutation. Similar mutations have been found in *P. aeruginosa* when exposed to ciprofloxacin under experimental evolution conditions (Wong et al., 2012). The fact that these mutations are located in a poly-G repeat region supports that this gene might have a specific high mutation rate as the consequence of polymerase slippage (Moxon et al., 2006). We hypothesize that this might be the reason why *orfN* mutants are detected in all replicates.

pmrAB is a two-component system involved in polymyxin resistance (Moskowitz et al., 2012). The system regulates the

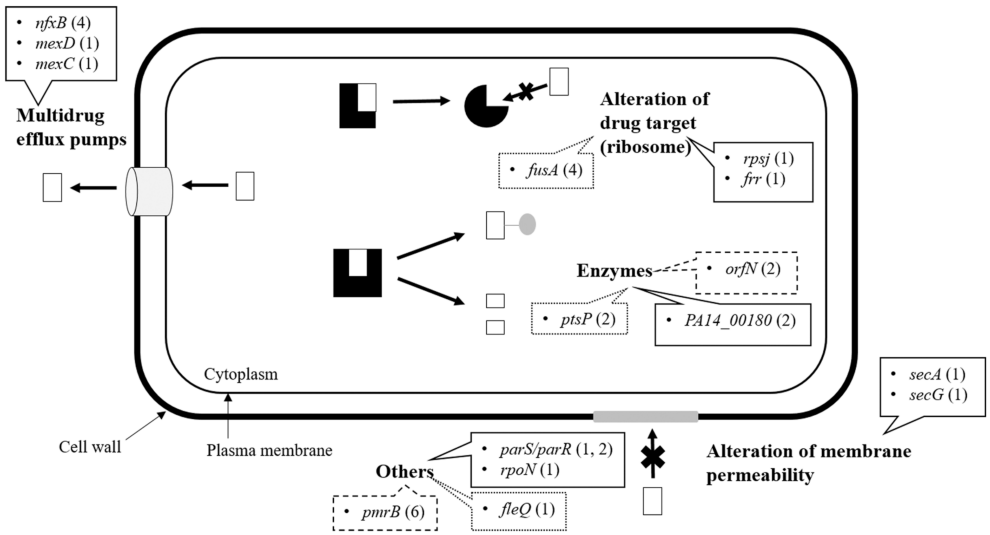

FIGURE 2 | Common and different genetic changes identified by whole-genome sequencing. Mutations detected by Illumina and verified by Sanger Sequencing in evolved *P. aeruginosa* PA14 at 32MIC tigecycline/tobramycin. Most mutations fell into the four major functional groups of resistance mechanism: multidrug efflux pumps, alteration of drug target, enzyme neutralization, and alteration of membrane permeability. Others that did not properly match any of these categories were entered the group "Others." The line type indicates the drug providing the selection pressure: continuous line, tigecycline; dots, tobramycin; dashes, both treatments. The figures after the genes refer to the number of different genetic changes found in each. These values also inform about the number of biological replicates in which a particular gene mutated, with the exceptions of *rpsJ* (only one type of mutation) and *orfN* (two different mutations that appeared in every evolved population).

expression of operons involved in the biosynthesis of lipid A with 4-aminoarabinose, which produces a more positively charged lipopolysaccharide, thus reducing the binding and the activity of cationic peptides (Gunn et al., 2000). Gain-of-function mutants with lipid A modifications can be selected for and are resistant to colistin (Fernández et al., 2010). Moreover, a recent evolution study of *P. aeruginosa* involving colistin gave rise to the selection of mutations in *pmrB* (Jochumsen et al., 2016), and it has been proposed that mutations in such regulatory elements may potentiate the effect of other mutations (Lind et al., 2015). Indeed, *pmrB* is involved in a wide range of antibiotic resistances, including those to quinolones, tigecycline, tobramycin and cationic peptides (Muller et al., 2011; Lopez-Causape et al., 2018b). In agreement with these data, the populations selected in our evolution experiment present a reduced susceptibility to colistin and polymixin B (**Table 1**).

Evolutionary Pathways Toward Tobramycin Resistance in *P. aeruginosa*

Having shown the common features of the evolutionary trajectories toward resistance to both test antibiotics, the specific pathways toward resistance to each were sought. Mutations in *fusA* and *ptsP* were found to be involved in evolution toward tobramycin resistance (Schurek et al., 2008; Wang et al., 2015; Feng et al., 2016). Together with the above mentioned *orfN* mutants, mutations in *fusA,* which codes for elongation factor G, may be envisaged as a first response to aminoglycosides (Wang et al., 2015; Feng et al., 2016) since they were observed in all evolved populations (**Figure 2**). Further, recent works have shown this type of mutations to be present in clinical isolates as well as in *in vitro* selected aminoglycoside resistant mutants (Bolard et al., 2018; Ibacache-Quiroga et al., 2018; Lopez-Causape et al., 2018b), reinforcing its role in the acquisition of aminoglycosides resistance. Although *mexXY* overexpression is considered a hallmark of aminoglycoside resistance development of CF chronic infections (Guenard et al., 2014), our data using PA14, as well as a recent experimental study using PAO1 (Lopez-Causape et al., 2018b), show that this does not necessarily always occurs, at least *in vitro*.

Consistent with a proposed role of *ptsP* in low-level tobramycin resistance (Schurek et al., 2008), the present results indicate that its mutation might also be important, because of its presence in 2 out of 4 tobramycin replicates. In fact, the MIC clearly increased when this mutation appeared alone (population 2, 8–24 μg/ml; **Figure 3** and **Supplementary Table S3**). Whether or not this type of mutation is selected for in clinical settings deserves further investigation. Finally, FleQ is a major flagellar regulator that has been found responsive to c-di-GMP and plays a role in biofilm formation (Hickman and Harwood, 2008; Jimenez et al., 2012; Matsuyama et al., 2016), although it has not been previously described to be involved in antibiotic resistance in planktonic cells.

It has been previously shown that mutations in at least 135 genes can render low-level aminoglycoside resistance in *P. aeruginosa* (Schurek et al., 2008). Despite the large number of possible combinations that might be selected along evolution under antibiotic selective pressure, we found that all four evolved populations contained mutants in *orfN*, *fusA* and *pmrB* and two of these evolved populations also presented mutants in *ptsP*. Further, recent work performed independently in another laboratory has also shown that *fusA* and *pmrB* mutants are

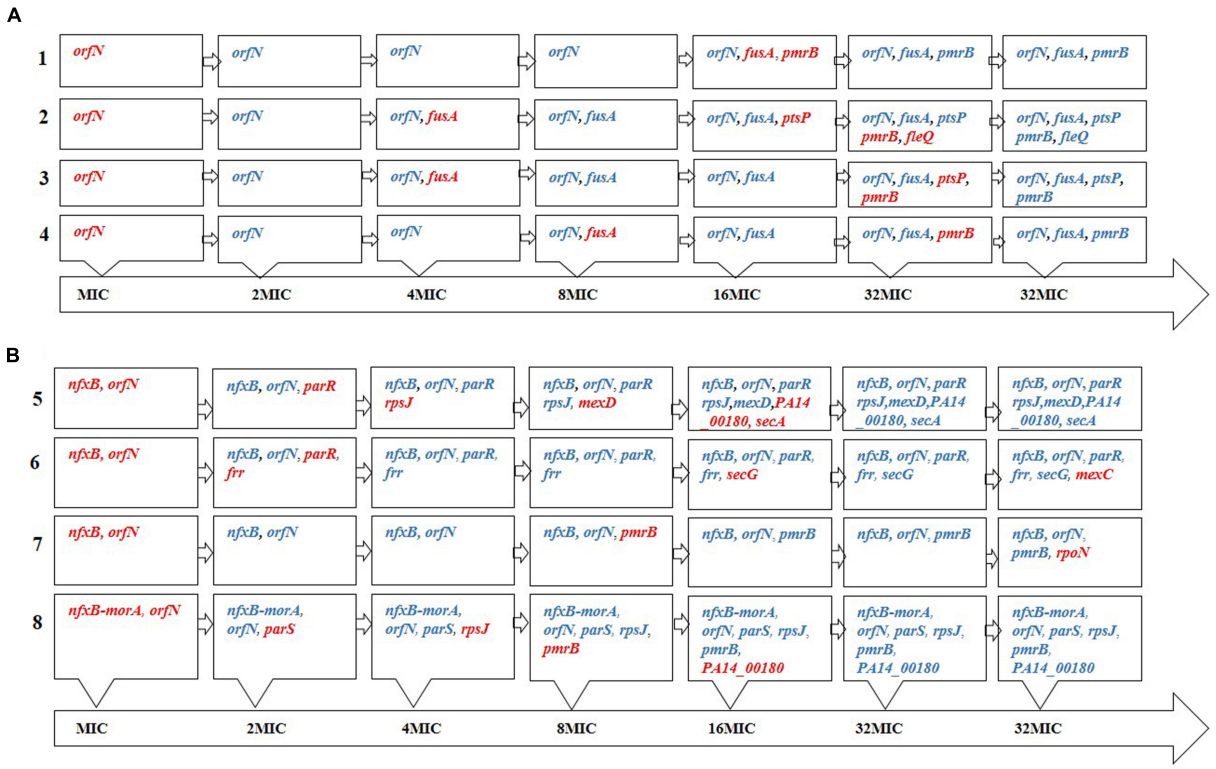

FIGURE 3 | Order of appearance of genetic changes. Order of appearance of **(A)** tobramycin and **(B)** tigecycline resistance mutations during the evolution process, as determined by PCR amplifications of known SNVs/MNVs in 32MIC populations. The names of the genes in red indicate that these mutations appeared in this step. Once a mutation appears it remains in the population until the end of the evolutionary period. We cannot discard that other mutations may have appeared and not fixed over the 35 days evolution period.

frequently selected in presence of aminoglycosides (Lopez-Causape et al., 2018b). Taking into consideration that the number of possible combinations of five mutations from the above mentioned 135 genes exceeds 3.4E8, our results indicate that *P. aeruginosa* mutation-driven resistance to tobramycin displays a certain degree of predictability.

Evolutionary Pathways Toward Tigecycline Resistance in *P. aeruginosa*

Pseudomonas aeruginosa PA14 evolving under selective pressure from tigecycline accumulated resistance mutations, indicating that, even in intrinsically resistant microorganisms, resistance may increase. Among the selected mutated genes, three – *rpsJ, parRS* and *nfxB* - have already been described involved in antibiotic resistance (a result that validates the present experimental strategy). The Val57 change in the 30S ribosomal protein S10 (*rpsJ*), which was present in two out of four replicates, is commonly seen in tetracycline-resistant clinical isolates of pathogens such as *Neisseria gonorrhoeae*, (Hu et al., 2005) while in *Klebsiella pneumoniae* different mutations in this gene have been related to tigecycline resistance (Ahn et al., 2016). Further, the two-component *parRS* system has been extensively examined in association with resistance to various drugs. In our experimental evolution assay, three out of the four tigecycline evolved populations presented mutations in this two-component system (**Figure 3**), which may be indicative of its importance in resistance. ParRS may promote multidrug resistance via three mechanisms: by inducing the expression of efflux pumps (such as MexXY-OprM), by repressing the expression of porins, and via LPS alteration (Muller et al., 2011).

The transcriptional repressor of the multidrug efflux pump MexCD-OprJ (Purssell and Poole, 2013), one of the most clinically important of all efflux pumps in *P. aeruginosa,* is coded for by *nfxB*. Loss-of-function mutations in this gene result in the pump's overexpression. Different mutations in *nfxB* were selected in all four replicates of the evolving populations, and most were fully inactivating mutations (a 377 bp deletion leading to a truncated protein; a transposition; and an 11 bp deletion). Although it has been suggested that the main efflux pump contributing to tigecycline resistance in *P. aeruginosa* is MexXY-OprM (Masuda et al., 2000a; Dean et al., 2003), the first step along the path to resistance in the present work was seen to be the selection of mutants able to extrude tigecycline via MexCD-OprJ. To ascertain whether or not *nfxB* selected mutations inactivate the repressor, hence allowing MexCD overexpression, one individual clone from each 5 days tigecycline evolved population was isolated in MHA. The presence of their corresponding *nfxB* mutations and the absence of *orfN* modification were confirmed by Sanger-sequencing. Expression

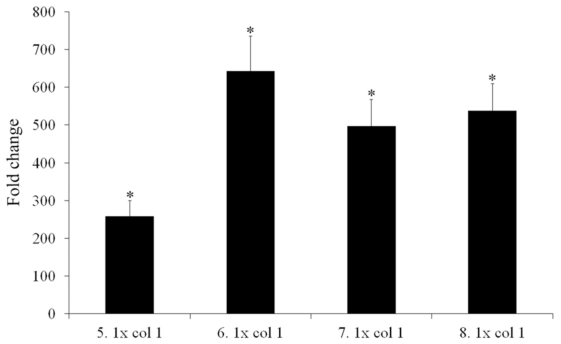

FIGURE 4 | *mexC* over-expression in tigecycline evolved individual clones. Four individual clones from the tigecycline evolved populations after 5 days of the experiment were isolated. The presence of the different *nfxB* mutations, as well as the absence of *orfN* mutations, were addressed. Fold changes were estimated with respect to the value given by *P. aeruginosa* PA14 strain. Error bars indicate standard deviations of the results from three independent experiments. Statistically significant differences ($p < 0.05$) in the level of expression of each population with respect to the wild type strain was evaluated using the Student's *T*-test and are highlighted with asterisks. 5.1–8.1: clones isolated from the populations evolved under tigecycline selective pressure at day five of evolution.

of *mexC* was measured in comparison with the one of the wild-type strain. As shown in **Figure 4**, all clones carrying *nfxB* mutations overexpressed *mexC*, confirming that these mutations inactivate, in different degree, the NfxB repressor. Two further mutations in each of the genes coding for the subunits of this efflux pump (*mexC* and *mexD*) were then selected for in populations 5 and 6 respectively. It may be that these mutations alter the specificity of MexCD-OprJ improving the capacity of the pump to extrude tigecycline. The MexD SNV (Phe608Cys) is located in one of the two large periplasmic loops known to be involved in substrate specificity (Elkins and Nikaido, 2002). In agreement with this, it has been described that mutations in AcrB, the *Enterobacteriaceae* ortholog of MexD, alter the pump's substrate profile (Blair et al., 2015). While a role for these mutations in reducing the susceptibility to tigecycline is a compelling hypothesis, it cannot be ruled out that mutations in *mexD* or *mexC* may compensate for any increased non-physiological extrusion of some important cellular metabolite by those mutants that overexpress this pump.

The present results indicate that tigecycline resistance might also come about through changes in the Sec system (*SecA* and *SecG*), likely via the modification of the permeability of the membrane and the impairment of tigecycline uptake. It has also been suggested that the Sec pathway could be involved in the translocation of components of multidrug efflux pumps across the bacterial membranes (Yoneyama et al., 2010; Akiba et al., 2013; Chaudhary et al., 2015), but it is unclear how changes in this translocation could increase resistance in the selected mutants. Tigecycline increased resistance may also come about through modifications of the σ_{54} RpoN factor that has been found to modulate *P. aeruginosa* virulence (Kazmierczak et al., 2005), bacterial tolerance to carbapenems (Viducic et al., 2016), susceptibility to quinolones (Viducic et al., 2007) and survival in the presence of tobramycin (Viducic et al., 2017). Both the Sec pathway and RpoN have been proposed as excellent targets in the search of novel antibiotics (Jin et al., 2016; Lloyd et al., 2017). Our findings suggest that tigecycline might select mutants presenting cross-resistance to these potential inhibitors, still under development. Mutations in the gene encoding the ribosome recycling factor *frr* are likely related to modifications in the tigecycline target (ribosome). Indeed, this particular mutation is located in the start codon (**Supplementary Table S1**), so it may affect the level of *frr*, impairing the steady state amount of active ribosomes. A similar situation might arise with *PA14_00180*, which codes for a putative rRNA small subunit methyltransferase, hence likely able to modify the ribosome.

All the changes seen during the evolution of the populations subjected to selective pressure from tigecycline indicate that evolutionary pathways toward tigecycline resistance present some common features. All four replicates present mutations in *orfN* and *nfxB* and mutations in *parRS* were selected in three out of the four populations. Mutations at *secAG, PA14_00180*, *rpsJ* or *mexCD* were selected in half (two) of the populations. This indicates that *P. aeruginosa* can develop resistance to tigecycline by following a limited number of different evolutionary trajectories, which share some specific type of mutations, suggesting a certain degree of determinism.

In addition of conferring resistance to the selecting antibiotics, the respective mutants also showed resistance to antibiotics belonging to other structural categories and with different targets. This might be explained, at least in part, via the important role that efflux pumps seem to play in the development of resistance. Indeed, all mutants selected in the presence of tigecycline showed mutations in *nfxB*, which would lead to the overexpression of MexCD-OprJ. In turn this might reduce susceptibility to quinolones, tetracyclines, beta-lactams and chloramphenicol (De Kievit et al., 2001) – phenotypes in agreement with the present data (**Table 1**). Moreover, all four population replicates analyzed showed reduced susceptibility to aminoglycosides. This phenotype may be explained in three populations as a result of the mutations selected for in the ParRS system, which is involved in modification of the lipopolysaccharide and in the regulation of the expression of MexXY-OprM, an efflux pump that contributes to aminoglycoside resistance in *P. aeruginosa* (Masuda et al., 2000a), and which is described as being regulated by the two-component system ParRS (Muller et al., 2011).

CONCLUSION

Experimental evolution approaches may allow determining basic aspects of evolution, among which knowing to what extent evolution (in our case of asexual organisms) can be predictable and hence deterministic or is basically stochastic (non-predictable) (Lassig et al., 2017), is notably interesting. This is particularly relevant in the case of antibiotic resistance, a field in which the implementation of novel therapeutic approaches is based in the assumption that evolution of antibiotic resistance can be largely predictable (Martinez et al., 2007), a feature that goes against most common views on evolution.

The present work provides information on the evolutionary trajectories leading to resistance to antibiotics belonging to different structural families but targeting the ribosome in *P. aeruginosa*. The results suggest that, although mutations in several genes may contribute to the development of antibiotic resistance (Fajardo et al., 2008; Tamae et al., 2008; Alvarez-Ortega et al., 2011; Martinez, 2012; Vestergaard et al., 2016; Lopez-Causape et al., 2018a), which may imply a large degree of stochasticity in the evolutionary trajectories, mutation-driven evolution toward resistance is partially deterministic, at least when bacteria grow in the same conditions. This opens up the possibility of predicting the appearance of antibiotic resistance (Martinez et al., 2007).

The selection of specific mutations depends primarily on the fitness of each mutant before/after selection and the rate of mutation supply for each of the mutations (Huseby et al., 2017; Hughes and Andersson, 2017). Recent work has shown that a high generalized mutation supply does not alter the types of mutations selected upon experimental evolution (Ibacache-Quiroga et al., 2018), in which case fitness costs, together with population bottlenecks (Vogwill et al., 2016), will be the main constrains for selecting some mutants over other mutations able of providing the same resistance phenotype. However, the situation might be different in the case of gene-specific high mutation rates, a situation that might have happened in the case of *orfN*. To note here that all mutations in *orfN* have been selected in a poly-G region, present in the *P. aeruginosa* PA14 strain, a situation that might increase the mutation rate as the consequence of polymerase slippage (Moxon et al., 2006). We hypothesize that this gene-specific high mutational supply might be in the basis of the presence of *orfN* mutants in all replicates. However, OrfN sequence is highly polymorphic in *P. aeruginosa* (Arora et al., 2004) becoming doubtful if these results can be extrapolated to strains with different *orfN* alleles.

The present results clearly show that challenging *P. aeruginosa* with tigecycline has clinically relevant consequences, despite this bacterial pathogen is considered to be intrinsically resistant to this antibiotic. Tigecycline is used for empiric treatment of Gram-negative infections, and in this type of patients, the main agent causing superinfection is *P. aeruginosa* (Garcia-Cabrera et al., 2010; Ulu-Kilic et al., 2015; Katsiari et al., 2016), which is most likely under tigecycline selection during treatment. Our results indicate that tigecycline selects mutants presenting reduced susceptibility to antibiotics of clinical value as aztreonam, ceftazidime, ciprofloxacin or aminoglycosides. Notably, these mutants also display an increased susceptibility to fosfomycin. Our results suggest that fosfomycin might be an antibiotic of choice for treating superinfections by *P. aeruginosa* in tigecycline treated patients. A recent work has shown that most antibiotic resistance mutations display strain-independent phenotypes (Knopp and Andersson, 2018). Nevertheless, it is also true that the current work has been performed with a single, model strain and, because of the diversity of *P. aeruginosa*, it would be important to examine additional strains to establish whether this phenotype depends on the genomic context or can be extrapolated to other *P. aeruginosa* clinical isolates.

Antibiotic resistance can be acquired by the acquisition of antibiotic resistance genes, which usually confer resistance to members of the same family of antibiotics. However, particularly relevant in the case of chronic infections is the selection of antibiotic resistant mutants. Our results indicate that antibiotic resistance mutations frequently have a pleiotropic effect, altering susceptibility to other drugs. In agreement with this is the fact that some of the mutations selected for by exposure to tobramycin or tigecycline have previously been described to confer resistance to other antibiotics belonging to different structural families.

AUTHOR CONTRIBUTIONS

FS-G and SH-A performed the experiments. JM and SH-A designed the work. All the authors participated in the interpretation of the results and in writing the article.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Ahn, C., Yoon, S. S., Yong, T. S., Jeong, S. H., and Lee, K. (2016). The resistance mechanism and clonal distribution of tigecycline-nonsusceptible *Klebsiella pneumoniae* isolates in Korea. *Yonsei Med. J.* 57, 641–646. doi: 10.3349/ymj.2016.57.3.641

Akiba, K., Ando, T., Isogai, E., Nakae, T., and Yoneyama, H. (2013). Tat pathway-mediated translocation of the Sec pathway substrate OprM, an outer membrane subunit of the resistance nodulation division xenobiotic extrusion pumps, in *Pseudomonas Aeruginosa*. *Chemotherapy* 59, 129–137. doi: 10.1159/000353894

Alvarez-Ortega, C., Wiegand, I., Olivares, J., Hancock, R. E., and Martinez, J. L. (2010). Genetic determinants involved in the susceptibility of *Pseudomonas*

aeruginosa to beta-lactam antibiotics. *Antimicrob. Agents Chemother.* 54, 4159–4167. doi: 10.1128/AAC.00257-10

Alvarez-Ortega, C., Wiegand, I., Olivares, J., Hancock, R. E., and Martinez, J. L. (2011). The intrinsic resistome of *Pseudomonas aeruginosa* to beta-lactams. *Virulence* 2, 144–146.

Appelbaum, P. C. (2012). 2012 and beyond: potential for the start of a second pre-antibiotic era? *J. Antimicrob. Chemother.* 67, 2062–2068. doi: 10.1093/jac/dks213dks213

Arora, S. K., Wolfgang, M. C., Lory, S., and Ramphal, R. (2004). Sequence polymorphism in the glycosylation island and flagellins of *Pseudomonas aeruginosa*. *J. Bacteriol.* 186, 2115–2122.

Blair, J. M., Bavro, V. N., Ricci, V., Modi, N., Cacciotto, P., Kleinekathfer, U., et al. (2015). AcrB drug-binding pocket substitution confers clinically relevant resistance and altered substrate specificity. *Proc. Natl. Acad. Sci. U.S.A.* 112, 3511–3516. doi: 10.1073/pnas.1419939112

Blake, K. L., and O'Neill, A. J. (2013). Transposon library screening for identification of genetic loci participating in intrinsic susceptibility and acquired resistance to antistaphylococcal agents. *J. Antimicrob. Chemother.* 68, 12–16. doi: 10.1093/jac/dks373

Bolard, A., Plesiat, P., and Jeannot, K. (2018). Mutations in gene *fusA1* as a novel mechanism of aminoglycoside resistance in clinical strains of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 62:e01835-17. doi: 10.1128/aac.01835-17

Brandis, G., Pietsch, F., Alemayehu, R., and Hughes, D. (2015). Comprehensive phenotypic characterization of rifampicin resistance mutations in *Salmonella* provides insight into the evolution of resistance in *Mycobacterium tuberculosis*. *J. Antimicrob. Chemother.* 70, 680–685. doi: 10.1093/jac/dku434

Breidenstein, E. B., Khaira, B. K., Wiegand, I., Overhage, J., and Hancock, R. E. (2008). Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. *Antimicrob. Agents Chemother.* 52, 4486–4491. doi: 10.1128/AAC.00222-08

Bryson, V., and Szybalski, W. (1952). Microbial selection. *Science* 116, 45–51. doi: 10.1126/science.116.3003.45

Cabot, G., Zamorano, L., Moya, B., Juan, C., Navas, A., Blazquez, J., et al. (2016). Evolution of *Pseudomonas aeruginosa* antimicrobial resistance and fitness under low and high mutation rates. *Antimicrob. Agents Chemother.* 60, 1767–1778. doi: 10.1128/AAC.02676-15

Chaudhary, A. S., Chen, W., Jin, J., Tai, P. C., and Wang, B. (2015). SecA: a potential antimicrobial target. *Future Med. Chem.* 7, 989–1007. doi: 10.4155/fmc.15.42

Cheer, S. M., Waugh, J., and Noble, S. (2003). Inhaled tobramycin (TOBI): a review of its use in the management of *Pseudomonas aeruginosa* infections in patients with cystic fibrosis. *Drugs* 63, 2501–2520.

De Kievit, T. R., Parkins, M. D., Gillis, R. J., Srikumar, R., Ceri, H., Poole, K., et al. (2001). Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* 45, 1761–1770.

Dean, C. R., Visalli, M. A., Projan, S. J., Sum, P. E., and Bradford, P. A. (2003). Efflux-mediated resistance to tigecycline (GAR-936) in *Pseudomonas aeruginosa* PAO1. *Antimicrob. Agents Chemother.* 47, 972–978.

Elkins, C. A., and Nikaido, H. (2002). Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of *Escherichia coli* is determined predominantly by two large periplasmic loops. *J. Bacteriol.* 184, 6490–6498.

Fajardo, A., Martinez-Martin, N., Mercadillo, M., Galan, J. C., Ghysels, B., Matthijs, S., et al. (2008). The neglected intrinsic resistome of bacterial pathogens. *PLoS One* 3:e1619. doi: 10.1371/journal.pone.0001619

Feng, Y., Jonker, M. J., Moustakas, I., Brul, S., and Ter Kuile, B. H. (2016). Dynamics of mutations during development of resistance by *Pseudomonas aeruginosa* against five antibiotics. *Antimicrob. Agents Chemother.* 60, 4229–4236. doi: 10.1128/AAC.00434-16

Fernandez, L., Alvarez-Ortega, C., Wiegand, I., Olivares, J., Kocincova, D., Lam, J. S., et al. (2013). Characterization of the polymyxin B resistome of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 57, 110–119. doi: 10.1128/AAC.01583-12

Fernández, L., Gooderham, W. J., Bains, M., McPhee, J. B., Wiegand, I., and Hancock, R. E. (2010). Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. *Antimicrob. Agents Chemother.* 54, 3372–3382. doi: 10.1128/AAC.00242-10

Garcia-Cabrera, E., Jimenez-Mejias, M. E., Gil Navarro, M. V., Gomez-Gomez, M. J., Ortiz-Leyba, C., Cordero, E., et al. (2010). Superinfection during treatment of nosocomial infections with tigecycline. *Eur. J. Clin. Microbiol. Infect. Dis.* 29, 867–871. doi: 10.1007/s10096-010-0942-y

Guenard, S., Muller, C., Monlezun, L., Benas, P., Broutin, I., Jeannot, K., et al. (2014). Multiple mutations lead to MexXY-OprM-dependent aminoglycoside resistance in clinical strains of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 58, 221–228. doi: 10.1128/aac.01252-13

Gunn, J. S., Ryan, S. S., Van Velkinburgh, J. C., Ernst, R. K., and Miller, S. I. (2000). Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar typhimurium. *Infect. Immun.* 68, 6139–6146.

Hernando-Amado, S., Sanz-Garcia, F., Blanco, P., and Martinez, J. L. (2017). Fitness costs associated with the acquisition of antibiotic resistance. *Essays Biochem.* 61, 37–48. doi: 10.1042/ebc20160057

Hickman, J. W., and Harwood, C. S. (2008). Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Mol. Microbiol.* 69, 376–389. doi: 10.1111/j.1365-2958.2008.06281.x

Hu, M., Nandi, S., Davies, C., and Nicholas, R. A. (2005). High-level chromosomally mediated tetracycline resistance in *Neisseria gonorrhoeae* results from a point mutation in the rpsJ gene encoding ribosomal protein S10 in combination with the mtrR and penB resistance determinants. *Antimicrob. Agents Chemother.* 49, 4327–4334. doi: 10.1128/AAC.49.10.4327-4334.2005

Hughes, D., and Andersson, D. I. (2017). Evolutionary trajectories to antibiotic resistance. *Annu. Rev. Microbiol.* 71, 579–596. doi: 10.1146/annurev-micro-090816-093813

Huseby, D. L., Pietsch, F., Brandis, G., Garoff, L., Tegehall, A., and Hughes, D. (2017). Mutation supply and relative fitness shape the genotypes of ciprofloxacin-resistant *Escherichia coli*. *Mol. Biol. Evol.* 34, 1029–1039. doi: 10.1093/molbev/msx052

Ibacache-Quiroga, C., Oliveros, J. C., Couce, A., and Blázquez, J. (2018). Parallel evolution of high-level aminoglycoside resistance in *Escherichia coli* under low and high mutation supply rates. *Front. Microbiol.* 9:427. doi: 10.3389/fmicb.2018.00427

Jimenez, P. N., Koch, G., Thompson, J. A., Xavier, K. B., Cool, R. H., and Quax, W. J. (2012). The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. *Microbiol. Mol. Biol. Rev.* 76, 46–65. doi: 10.1128/MMBR.05007-11

Jin, J., Hsieh, Y. H., Cui, J., Damera, K., Dai, C., Chaudhary, A. S., et al. (2016). Using chemical probes to assess the feasibility of targeting seca for developing antimicrobial agents against gram-negative bacteria. *ChemMedChem* 11, 2511–2521. doi: 10.1002/cmdc.201600421

Jochumsen, N., Marvig, R. L., Damkiaer, S., Jensen, R. L., Paulander, W., Molin, S., et al. (2016). The evolution of antimicrobial peptide resistance in *Pseudomonas aeruginosa* is shaped by strong epistatic interactions. *Nat. Commun.* 7:13002. doi: 10.1038/ncomms13002

Katsiari, M., Ntorlis, K., Nteves, I., Roussou, Z., Platsouka, E. D., and Maguina, A. (2016). Characteristics of superinfections during treatment with tigecycline. *J. Chemother.* 28, 110–115. doi: 10.1080/1120009x.2015.1118184

Kazmierczak, M. J., Wiedmann, M., and Boor, K. J. (2005). Alternative sigma factors and their roles in bacterial virulence. *Microbiol. Mol. Biol. Rev.* 69, 527–543. doi: 10.1128/MMBR.69.4.527-543.2005

Knopp, M., and Andersson, D. I. (2018). Predictable phenotypes of antibiotic resistance mutations. *mBio* 9:e00770-18. doi: 10.1128/mBio.00770-18

Kotra, L. P., Haddad, J., and Mobashery, S. (2000). Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. *Antimicrob. Agents Chemother.* 44, 3249–3256.

Lassig, M., Mustonen, V., and Walczak, A. M. (2017). Predicting evolution. *Nat. Ecol. Evol.* 1:77. doi: 10.1038/s41559-017-0077

Levin, B. R., and Rozen, D. E. (2006). Non-inherited antibiotic resistance. *Nat. Rev. Microbiol.* 4, 556–562.

Lind, P. A., Farr, A. D., and Rainey, P. B. (2015). Experimental evolution reveals hidden diversity in evolutionary pathways. *eLife* 4:e07074. doi: 10.7554/eLife.07074

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402–408.

Lloyd, M. G., Lundgren, B. R., Hall, C. W., Gagnon, L. B., Mah, T. F., Moffat, J. F., et al. (2017). Targeting the alternative sigma factor RpoN to combat virulence in *Pseudomonas aeruginosa*. *Sci. Rep.* 7:12615. doi: 10.1038/s41598-017-12667-y

Lopez-Causape, C., Cabot, G., Del Barrio-Tofino, E., and Oliver, A. (2018a). The versatile mutational resistome of *Pseudomonas aeruginosa*. *Front. Microbiol.* 9:685. doi: 10.3389/fmicb.2018.00685

Lopez-Causape, C., Rubio, R., Cabot, G., and Oliver, A. (2018b). Evolution of the *Pseudomonas aeruginosa* aminoglycoside mutational resistome in vitro and in the cystic fibrosis setting. *Antimicrob. Agents Chemother.* 62:e02583-17. doi: 10.1128/aac.02583-17

Martinez, J. L. (2012). The antibiotic resistome: challenge and opportunity for therapeutic intervention. *Future Med. Chem.* 4, 347–359. doi: 10.4155/fmc.12.2

Martinez, J. L., and Baquero, F. (2000). Mutation frequencies and antibiotic resistance. *Antimicrob. Agents Chemother.* 44, 1771–1777.

Martinez, J. L., Baquero, F., and Andersson, D. I. (2007). Predicting antibiotic resistance. *Nat. Rev. Microbiol.* 5, 958–965.

Martinez, J. L., Baquero, F., and Andersson, D. I. (2011). Beyond serial passages: new methods for predicting the emergence of resistance to novel antibiotics. *Curr. Opin. Pharmacol.* 11, 439–445. doi: 10.1016/j.coph.2011.07.005

Martinez, J. L., Coque, T. M., and Baquero, F. (2015). What is a resistance gene? Ranking risk in resistomes. *Nat. Rev. Microbiol.* 13, 116–123. doi: 10.1038/nrmicro3399

Martinez, J. L., Fajardo, A., Garmendia, L., Hernandez, A., Linares, J. F., Martinez-Solano, L., et al. (2009). A global view of antibiotic resistance. *FEMS Microbiol. Rev.* 33, 44–65. doi: 10.1111/j.1574-6976.2008.00142.x

Martinez, J. L., and Rojo, F. (2011). Metabolic regulation of antibiotic resistance. *FEMS Microbiol. Rev.* 35, 768–789. doi: 10.1111/j.1574-6976.2011.00282.x

Martinez-Solano, L., Macia, M. D., Fajardo, A., Oliver, A., and Martinez, J. L. (2008). Chronic *Pseudomonas aeruginosa* infection in chronic obstructive pulmonary disease. *Clin. Infect. Dis.* 47, 1526–1533. doi: 10.1086/593186

Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H., and Nishino, T. (2000a). Contribution of the MexX-MexY-oprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 44, 2242–2246.

Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H., and Nishino, T. (2000b). Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 44, 3322–3327.

Matsuyama, B. Y., Krasteva, P. V., Baraquet, C., Harwood, C. S., Sondermann, H., and Navarro, M. V. (2016). Mechanistic insights into c-di-GMP-dependent control of the biofilm regulator FleQ from *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 113, E209–E218. doi: 10.1073/pnas.1523148113

Meftahi, N., Namouchi, A., Mhenni, B., Brandis, G., Hughes, D., and Mardassi, H. (2016). Evidence for the critical role of a secondary site rpoB mutation in the compensatory evolution and successful transmission of an MDR tuberculosis outbreak strain. *J. Antimicrob. Chemother.* 71, 324–332. doi: 10.1093/jac/dkv345

Moskowitz, S. M., Brannon, M. K., Dasgupta, N., Pier, M., Sgambati, N., Miller, A. K., et al. (2012). PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. *Antimicrob. Agents Chemother.* 56, 1019–1030. doi: 10.1128/AAC.05829-11

Moxon, R., Bayliss, C., and Hood, D. (2006). Bacterial contingency loci: the role of simple sequence DNA repeats in bacterial adaptation. *Annu. Rev. Genet.* 40, 307–333. doi: 10.1146/annurev.genet.40.110405.090442

Mulet, X., Maciá, M. D., Mena, A., Juan, C., Pérez, J. L., and Oliver, A. (2009). Azithromycin in *Pseudomonas aeruginosa* biofilms: bactericidal activity and selection of nfxB mutants. *Antimicrob. Agents Chemother.* 53, 1552–1560. doi: 10.1128/aac.01264-08

Muller, C., Plesiat, P., and Jeannot, K. (2011). A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and beta-lactams in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 55, 1211–1221. doi: 10.1128/aac.01252-10

Palmer, G. C., and Whiteley, M. (2015). Metabolism and pathogenicity of *Pseudomonas aeruginosa* infections in the lungs of individuals with cystic fibrosis. *Microbiol. Spectr.* 3. doi: 10.1128/microbiolspec.MBP-0003-2014

Pankey, G. A. (2005). Tigecycline. *J. Antimicrob. Chemother.* 56, 470–480. doi: 10.1093/jac/dki248

Pietsch, F., Bergman, J. M., Brandis, G., Marcusson, L. L., Zorzet, A., Huseby, D. L., et al. (2017). Ciprofloxacin selects for RNA polymerase mutations with pleiotropic antibiotic resistance effects. *J. Antimicrob. Chemother.* 72, 75–84. doi: 10.1093/jac/dkw364

Purssell, A., and Poole, K. (2013). Functional characterization of the NfxB repressor of the mexCD-oprJ multidrug efflux operon of *Pseudomonas aeruginosa*. *Microbiology* 159(Pt 10), 2058–2073. doi: 10.1099/mic.0.069286-0

Roca, I., Akova, M., Baquero, F., Carlet, J., Cavaleri, M., Coenen, S., et al. (2015). The global threat of antimicrobial resistance: science for intervention. *New Microbes New Infect.* 6, 22–29. doi: 10.1016/j.nmni.2015.02.007

Schirm, M., Arora, S. K., Verma, A., Vinogradov, E., Thibault, P., Ramphal, R., et al. (2004). Structural and genetic characterization of glycosylation of type a flagellin in *Pseudomonas aeruginosa*. *J. Bacteriol.* 186, 2523–2531.

Schurek, K. N., Marr, A. K., Taylor, P. K., Wiegand, I., Semenec, L., Khaira, B. K., et al. (2008). Novel genetic determinants of low-level aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 52, 4213–4219. doi: 10.1128/AAC.00507-08

Scortti, M., Lacharme-Lora, L., Wagner, M., Chico-Calero, I., Losito, P., and Vázquez-Boland, J. A. (2006). Coexpression of virulence and fosfomycin susceptibility in Listeria: molecular basis of an antimicrobial in vitro-in vivo paradox. *Nat. Med.* 12, 515–517.

Shcherbakov, D., Akbergenov, R., Matt, T., Sander, P., Andersson, D. I., and Böttger, E. C. (2010). Directed mutagenesis of *Mycobacterium smegmatis* 16S rRNA to reconstruct the in-vivo evolution of aminoglycoside resistance in *Mycobacterium tuberculosis*. *Mol. Microbiol.* 77, 830–840. doi: 10.1111/j.1365-2958.2010.07218.x

Silby, M. W., Winstanley, C., Godfrey, S. A., Levy, S. B., and Jackson, R. W. (2011). *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiol. Rev.* 35, 652–680. doi: 10.1111/j.1574-6976.2011.00269.x

Tamae, C., Liu, A., Kim, K., Sitz, D., Hong, J., Becket, E., et al. (2008). Determination of antibiotic hypersensitivity among 4,000 single-gene-knockout mutants of *Escherichia coli*. *J. Bacteriol.* 190, 5981–5988. doi: 10.1128/JB.01982-07

Toprak, E., Veres, A., Michel, J. B., Chait, R., Hartl, D. L., and Kishony, R. (2011). Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nat. Genet.* 44, 101–105. doi: 10.1038/ng.1034

Tummler, B., Wiehlmann, L., Klockgether, J., and Cramer, N. (2014). Advances in understanding *Pseudomonas*. *F1000Prime Rep.* 6:9. doi: 10.12703/P6-9

Turrientes, M. C., Baquero, F., Levin, B. R., Martinez, J. L., Ripoll, A., Gonzalez-Alba, J. M., et al. (2013). Normal mutation rate variants arise in a Mutator (Mut S) *Escherichia coli* population. *PLoS One* 8:e72963. doi: 10.1371/journal.pone.0072963

Ulu-Kilic, A., Alp, E., Altun, D., Cevahir, F., Kalın, G., and Demiraslan, H. (2015). Increasing frequency of *Pseudomonas aeruginosa* infections during tigecycline use. *J. Infect. Dev. Ctries.* 9, 309–312. doi: 10.3855/jidc.4700

Vestergaard, M., Leng, B., Haaber, J., Bojer, M. S., Vegge, C. S., and Ingmer, H. (2016). Genome-wide identification of antimicrobial intrinsic resistance determinants in *Staphylococcus aureus*. *Front. Microbiol.* 7:2018. doi: 10.3389/fmicb.2016.02018

Viducic, D., Murakami, K., Amoh, T., Ono, T., and Miyake, Y. (2016). RpoN modulates carbapenem tolerance in *Pseudomonas aeruginosa* through *Pseudomonas* quinolone signal and PqsE. *Antimicrob. Agents Chemother.* 60, 5752–5764. doi: 10.1128/aac.00260-16

Viducic, D., Murakami, K., Amoh, T., Ono, T., and Miyake, Y. (2017). RpoN promotes *Pseudomonas aeruginosa* survival in the presence of tobramycin. *Front. Microbiol.* 8:839. doi: 10.3389/fmicb.2017.00839

Viducic, D., Ono, T., Murakami, K., Katakami, M., Susilowati, H., and Miyake, Y. (2007). rpoN gene of *Pseudomonas aeruginosa* alters its susceptibility to quinolones and carbapenems. *Antimicrob. Agents Chemother.* 51, 1455–1462. doi: 10.1128/AAC.00348-06

Vogne, C., Aires, J. R., Bailly, C., Hocquet, D., and Plesiat, P. (2004). Role of the multidrug efflux system MexXY in the emergence of moderate resistance to aminoglycosides among *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *Antimicrob. Agents Chemother.* 48, 1676–1680.

Vogwill, T., Phillips, R. L., Gifford, D. R., and MacLean, R. C. (2016). Divergent evolution peaks under intermediate population bottlenecks during bacterial experimental evolution. *Proc. Biol. Sci.* 283:20160749. doi: 10.1098/rspb.2016.0749

Wang, D., Dorosky, R. J., Han, C. S., Lo, C. C., Dichosa, A. E., Chain, P. S., et al. (2015). Adaptation genomics of a small-colony variant in a *Pseudomonas chlororaphis* 30-84 biofilm. *Appl. Environ. Microbiol.* 81, 890–899. doi: 10.1128/AEM.02617-14

Westbrock-Wadman, S., Sherman, D. R., Hickey, M. J., Coulter, S. N., Zhu, Y. Q., Warrener, P., et al. (1999). Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. *Antimicrob. Agents Chemother.* 43, 2975–2983.

Wong, A., Rodrigue, N., and Kassen, R. (2012). Genomics of adaptation during experimental evolution of the opportunistic pathogen *Pseudomonas aeruginosa*. *PLoS Genet.* 8:e1002928. doi: 10.1371/journal.pgen.1002928

Yoneyama, H., Akiba, K., Hori, H., Ando, T., and Nakae, T. (2010). Tat pathway-mediated translocation of the sec pathway substrate protein MexA, an inner membrane component of the MexAB-OprM xenobiotic extrusion pump in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 54, 1492–1497. doi: 10.1128/aac.01495-09

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Artículo III

Antibiotic resistance evolution is contingent on the quorum-sensing response in *Pseudomonas aeruginosa*

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Numerosos trabajos han aludido al grado de determinismo que posee la evolución de la resistencia adquirida a antibióticos en bacterias, en contraposición al concepto estocástico que generalmente se tiene del proceso evolutivo. Este constreñimiento podría ayudar palpablemente a su predicción, mediante el análisis riguroso de los factores que lo encorsetan. Entre estos factores se encuentra la epistasia, que hasta la fecha se ha estudiado principalmente entre genes y/o mutaciones de resistencia. Sin embargo, un campo prácticamente incólume es el de las interacciones epistáticas entre mutaciones eco-adaptativas y de resistencia a los antibióticos.

Este artículo aspira a enmendar esa ausencia, mediante el parangón de las trayectorias evolutivas seguidas durante una ALE en presencia de tobramicina y tigeciclina por un mutante de *P. aeruginosa* PA14 defectivo en el regulador de QS LasR y su cepa parental. El mutante en *lasR* fue escogido dada su repetidamente constatada selección *in vitro* como mecanismo de adaptación al medio de crecimiento sin antibióticos, en contraste a su total incomparecencia en las poblaciones que evolucionaron en presencia de éstos, en las ALEs reflejadas en el Artículo II. Esto nos llevó a hipotetizar la existencia de epistasia negativa entre ciertas mutaciones de resistencia y la de *lasR*, lo que explicaría la incompatibilidad de su selección conjunta en presencia de antibiótico. Así las cosas, la ALE y posterior secuenciación de los genomas de las poblaciones desvelaron diferencias entre las trayectorias evolutivas trazadas por la cepa mutante en *lasR* y la silvestre: la resistencia a las drogas de selección, el fenotipo de sensibilidad a otros antimicrobianos y las mutaciones seleccionadas en cada fondo genético difirieron.

Posteriormente se introdujo el alelo defectivo de *lasR* en mutantes resistentes obtenidos de la ALE de la cepa PA14, con vistas a ahondar en las posibles interacciones epistáticas entre las mutaciones que contuvieran y la de *lasR*. Por un lado, se recrearon mutantes de tiempo final, provistos de una batería de mutaciones representativa; y por otro lado un mutante del primer paso, para dilucidar si la mutación inicial en *orfN*, presente en todas las réplicas, condicionó las rutas evolutivas. De este modo se observó que existía epistasia negativa entre algunos determinantes de resistencia y *lasR*, lo que llevaba a contingencia recíproca: la selección temprana de mutantes en este regulador cercena el repertorio de rumbos hacia la resistencia, mientras que la primeriza aparición de ciertas mutaciones de resistencia veta la selección de mutantes defectivos en *lasR* en presencia de antibióticos. En resumen, estos resultados recalcan la indúbita influencia de la epistasia y la contingencia en la evolución de la resistencia en *P. aeruginosa*.

Aportaciones específicas:

Trabajo experimental: Sanz-García, F. y Hernando-Amado, S. contribuyeron a la labor experimental. Concretamente, mi participación se centró en la elaboración de los mutantes poseedores del alelo defectivo en *lasR*, en su análisis y en la interpretación de los resultados.

Elaboración del manuscrito: todos los autores contribuyeron a la escritura y corrección del manuscrito.

Antibiotic Resistance Evolution Is Contingent on the Quorum-Sensing Response in *Pseudomonas aeruginosa*

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Abstract

Different works have explored independently the evolution toward antibiotic resistance and the role of eco-adaptive mutations in the adaptation to a new habitat (as the infected host) of bacterial pathogens. However, knowledge about the connection between both processes is still limited. We address this issue by comparing the evolutionary trajectories toward antibiotic resistance of a *Pseudomonas aeruginosa lasR* defective mutant and its parental wild-type strain, when growing in presence of two ribosome-targeting antibiotics. Quorum-sensing *lasR* defective mutants are selected in *P. aeruginosa* populations causing chronic infections. Further, we observed they are also selected in *vitro* as a first adaptation for growing in culture medium. By using experimental evolution and whole-genome sequencing, we found that the evolutionary trajectories of *P. aeruginosa* in presence of these antibiotics are different in *lasR* defective and in wild-type backgrounds, both at the phenotypic and the genotypic levels. Recreation of a set of mutants in both genomic backgrounds (either wild type or *lasR* defective) allowed us to determine the existence of negative epistatic interactions between *lasR* and antibiotic resistance determinants. These epistatic interactions could lead to mutual contingency in the evolution of antibiotic resistance when *P. aeruginosa* colonizes a new habitat in presence of antibiotics. If *lasR* mutants are selected first, this would constraint antibiotic resistance evolution. Conversely, when resistance mutations (at least those studied in the present work) are selected, *lasR* mutants may not be selected in presence of antibiotics. These results underlie the importance of contingency and epistatic interactions in modulating antibiotic resistance evolution.

Key words: **antibiotic resistance, *Pseudomonas aeruginosa*, quorum sensing, epistasis, *lasR*.**

Introduction

Conventional wisdom establishes that evolution is sensitive to many stochastic influences so that it should be, mainly, unpredictable (Gould 1994). Nevertheless, many examples of convergent adaptation to similar conditions have been reported (Blount et al. 2018). Further, different studies based on experimental evolution have shown that the number of different evolutionary trajectories that bacterial populations submitted to the same selective pressure can follow is limited, hence presenting some degree of determinism (Barrick et al. 2009; Toprak et al. 2011; Lazar et al. 2013; Ibacache-Quiroga et al. 2018; Sanz-Garcia et al. 2018b). By combining experimental data and mathematical modeling (Huseby et al. 2017), as well as by blending information from molecular epidemiology studies with genetic reconstructions (Shcherbakov et al. 2010), different studies have reported also that mutation-driven bacterial evolution may present (at least in occasions) a certain degree of predictability.

In the case of antibiotic resistance (AR), the main factors that seem to constraint evolution are the mutation rate, the level of resistance and the impact of each mutation in bacterial fitness, and the strength of selection pressure (Hughes and Andersson 2017). Other elements that also restrict evolution are population bottlenecks, clonal interference, cross-selection, compensatory evolution, collateral sensitivity, and epistasis (Weinreich 2005; de Visser and Krug 2014; Szamecz et al. 2014; Maddamsetti et al. 2015; Gifford et al. 2016; Imamovic et al. 2018; Nichol et al. 2019; Rosenkilde et al. 2019).

Fitness costs associated with AR, when acquired by mutation, depend on the effect that resistance mutations have on bacterial physiology (Sander et al. 2002; Linares et al. 2005; Baquero et al. 2009; Fajardo et al. 2009; Andersson and Hughes 2010; Skurnik et al. 2013; Hernando-Amado et al. 2017). Conversely, the basal bacterial physiology, which can vary in different strains, may differentially affect fitness costs. This means that the type of resistant mutants present in a given genetic background might be restricted because of epistatic interactions (Baquero 2013; Agnello et al. 2016; Fuzi 2016), which may affect their level of resistance, their relative fitness, or both (Vogwill et al. 2016; Knopp and Andersson 2018). This situation is particularly evident in the case of combinations of AR mutations. The cumulative selection of AR mutations at different *loci* introduces historical constraints that reduce the variety of pathways leading to AR (Weinreich 2005; Weinreich et al. 2006; Schenk and de Visser 2013; Jochumsen et al. 2016). Further, the order in which mutations are selected is also important for the final outcome (Novais et al. 2010), which indicates the relevance of contingency in the evolution of AR.

Experimental evolution assays have been used to determine the importance of epistasis underlying evolutionary trajectories (Salverda et al. 2011; Tenaillon et al. 2012; Kryazhimskiy et al. 2014). Most studies in the field have analyzed epistasic interactions among different AR mutations (Trindade et al. 2009; Ward et al. 2009; Schenk et al. 2013; Wong 2017; Knopp and Andersson 2018). However, the effect of eco-adaptive mutations in shaping the evolution of AR through epistatic interactions has not been studied in such detail. Nevertheless, the pleiotropic effects caused by adaptive mutations, selected in the absence of selective pressure, but able to confer AR, have been explored (Rodriguez-Verdugo et al. 2013; Knoppel et al. 2017), indicating that AR can be interlinked with general aspects of the bacterial physiology (Martinez and Rojo 2011; Baquero and Martinez 2017).

It is worth mentioning that understanding the effect of eco-adaptive mutations in AR is of relevance for human health. Indeed, the study of *Pseudomonas aeruginosa* isolates producing chronic infections in cystic fibrosis (CF) or in chronic obstructive disease patients shows they present common patterns of evolution (Martinez-Solano et al. 2008; Huse et al. 2010; Yang et al. 2011). To what extent eco-adaptive evolution of bacteria, when facing a new environment, may influence the evolutionary trajectories toward AR of these host-adapted strains is a topic that remains to be analyzed in depth.

In a former study, we have applied adaptive laboratory evolution (ALE) and whole-genome sequencing (WGS) approaches to decipher the evolutionary trajectories of *P. aeruginosa* submitted to selective pressure by the ribosome-targeting antibiotics tobramycin or tigecycline (Sanz-Garcia et al. 2018b). We ascertained that the evolutionary trajectories of culture replicates presented common genetic elements, indicating that evolution toward AR has some degree of determinism. In addition, we found that mutants presenting two base pairs deletion (GA) in *lasR* (hereafter dubbed as *lasR**) were selected in all the control populations evolved in the absence of antibiotics. Opposite to this situation and despite the *lasR** mutation is likely an eco-adaptive mutation that improves the capability of *P. aeruginosa* for growing in the culture medium, *lasR** mutants were not selected in the populations challenged with antibiotics. This feature suggests the existence of epistatic effects between *lasR** and AR mutaions.

LasR is one of the regulators that, together with RhlR and PqsR, controls the three different, but interconnected, regulatory networks which hierarchically regulate the *P. aeruginosa* quorum-sensing (QS) response (Kiratisin et al. 2002). Although this cell-to-cell communication system constitutes a cooperative genetic program that enhances the colonizing ability of microbial population in the infected host (Williams et al. 2007), *lasR* cheaters are selected in vitro and are frequently found in clinical isolates of *P. aeruginosa* from patients with CF (Sandoz et al. 2007). In addition, this genotype enhances the fitness of the entire population during in vitro culture and in a mouse lung infection model (Smith et al. 2006; Sandoz et al. 2007; Hoffman et al. 2009; Zhao et al. 2016). Our results show the existence of negative epistasis between *lasR** and AR mutations. Further, they suggest that *lasR* mutations, besides their potential role for improving *P. aeruginosa* fitness during chronic infections, may also be relevant in shaping the evolution toward AR of *P. aeruginosa*.

Results and Discussion

lasR Deficient Mutants Are Early Selected during *P. aeruginosa* ALE

The evolutionary trajectories of *P. aeruginosa* in the presence of either tobramycin or tigecycline have been previously determined (Sanz-Garcia et al. 2018b). In this study, we found that *P. aeruginosa* mutants presenting two base pairs deletion in *lasR*, which produces a 223 frameshift in LasR, were selected in all control populations grown in absence of antibiotic selection. No mutation in this gene was found in any of the populations evolved under antibiotics selective pressure. To analyze the kinetics of emergence of the *lasR** mutation, this gene was Sanger-sequenced from the evolved populations at days 5, 10, 15, 20, 25, and 30 of evolution. All control populations presented the aforementioned genetic deletion at all evolutionary stages, whereas this mutation was not detected in any of the evolution steps of the populations challenged with either tobramycin or tigecycline. These results indicate that *lasR** mutants were early selected, likely as a mechanism of adaption for growing in the laboratory growth medium (see below), but this mutation may not provide a fitness gain when *P. aeruginosa* grows in presence of either tobramycin or tigecycline.

Characterization of the *lasR** Mutant

The aforementioned *lasR** mutation was detected at the population level. To further study the effect of this mutation in the behavior of *P. aeruginosa*, a clone from an evolved control population, at day 5 of evolution and containing the *lasR** mutation, was selected and sequenced. The clone contained the two base pairs deletion leading to the 223-frameshift mutation in LasR previously detected in the whole population. No further mutation was found in the genome of the *lasR** mutant. To ascertain the effect of this mutation on the regulatory activity of LasR, the expression of a set of QS-regulated genes was measured in *lasR** and compared with that of the wild-type parental strain PA14 during stationary phase, using the oligonucleotides described in supplementary table S1, Supplementary Material online. As shown in supplementary figure S1, Supplementary Material online, expression of QS-regulated genes was impaired in the *lasR** mutant. In addition, QS-related phenotypes were affected in this mutant: a reduced swarming motility and impaired biofilm formation (supplementary fig. S2, Supplementary Material online). Altogether these results indicate that the *lasR** mutation impairs *P. aeruginosa* QS response. It has been stated that triggering the QS response consumes an important amount of *P. aeruginosa* metabolic resources (Ruparell et al. 2016), in which case the *lasR** mutant should be fitter than its wild-type parental, at least at stationary growth phase, when the QS response is triggered. In agreement with this hypothesis, the *lasR** mutant reaches higher optical density at stationary

phase (maximum optical density of 0.84 and 0.71 in *lasR** and PA14, respectively; $P < 0.05$), while no significant differences in exponential growth rate were observed (supplementary fig. S3, Supplementary Material online). This result supports the notion that *P. aeruginosa lasR** mutants are selected in the laboratory growth medium because they reach higher cell densities (in this respect they are fitter) than the wild-type parental strain in these growing conditions, at least when antibiotics are absent. In addition, this emphasizes the metabolic burden imposed by the QS response and the growth advantage of deactivating this cellular response which, while not leading to faster growth, allows to reach higher cell densities.

A trivial explanation to the absence of *lasR** mutants when bacteria evolve in presence of antibiotics might be that this mutation increases the susceptibility of *P. aeruginosa* to the antibiotics used for selection, tobramycin and tigecycline. To ascertain this possibility, the susceptibility of both strains, *lasR** mutant and PA14 parental, to a set of antibiotics, was determined. As shown in table 1, the MICs of tigecycline and of tobramycin were exactly the same for both strains. This result indicates that the absence of *lasR** mutants in the antibiotics evolved populations is not merely a consequence of a higher susceptibility of these mutants to the selective agents that bacteria were confronted with along experimental evolution.

Evolutionary Landscapes of *lasR** *P. aeruginosa* toward AR

Given the above described results, it is possible that the presence of the *lasR** mutation modifies the evolutionary landscapes toward AR of *P. aeruginosa*. This may imply AR evolution to be contingent on the *P. aeruginosa* QS response. To analyze this possibility, 12 biological replicates of the *lasR** mutant were submitted to ALE (fig. 1), under the same conditions previously used for the wild-type PA14 strain (Sanz-Garcia et al. 2018b). These comprise four control populations (populations 1–4), four challenged with tigecycline (populations 5–8), and four challenged with tobramycin (populations 9–12). The MICs for each population of the antibiotic used for selection were determined every 5 days during the 30 days of the experiment (fig. 1). As shown in figure 2, a progressive increase in the level of resistance was observed for both antibiotics and in both genetic backgrounds, *lasR** and the previously analyzed PA14 strain (Sanz-Garcia et al. 2018b), suggesting either accumulation of consecutive mutations after each increase in antibiotic concentration or the displacement of low-level resistant mutants by higher-level resistant ones. The pattern of *lasR** phenotypic evolution was compared with that previously determined for the wild-type strain PA14 (Sanz-Garcia et al. 2018b). As shown in figure 2, all four *lasR** populations submitted to tobramycin selective pressure reached higher levels of resistance than the wild-type populations. The opposite was found in the case of tigecycline: most of the *lasR** evolved populations presented a lower MIC than the PA14 evolved populations. This indicates that the *lasR** mutation modifies the phenotypic evolutionary trajectories of *P. aeruginosa* when submitted to antibiotic selective pressure (fig. 3). To discard the possibility that the phenotypic differences observed might be due to a transient phenotypic adaptation to the presence of an antibiotic rather than to mutations (Levin and Rozen 2006; Martinez et al. 2009; Martinez and Rojo 2011), the evolved populations were sequentially subcultured in the absence of selection pressure (three sequential passages in medium without antibiotics) and the MICs again determined. No changes in MICs were detected, indicating that the observed phenotypes were due to inherited changes.

Table 1. Susceptibility to Antibiotics of the Studied Strains.

Strains	MIC (μg/ml)							
	Tgc	Tob	S	Ak	Cip	Atm	Caz	F
PA14	4	1	16	3	0.094	1	0.75	48
*lasR**	4	1	16	3	0.094	1.5	1	48
PA14-2[a]	64	24	192	256	0.38	3	2	3
PA14-2 *lasR**	96	16	256	96	0.38	3	2	3
PA14-5[b]	≥256	6	48	24	0.25	4	2	6
PA14-5 *lasR**	≥256	3	32	16	0.38	3	3	32

NOTE.—Tgc, tigecycline; Tob, tobramycin; S, streptomycin; Ak, amikacin; Cip, ciprofloxacin; Atm, aztreonam; Caz, ceftazidime; F, fosfomycin.
[a]Clone presenting mutations in *orfN*, *fusA*, *ptsP*, *fleQ*, and *pmrB*.
[b]Clone presenting mutations in *nfxB*, *orfN*, *mexD*, *rpsJ*, *parR*, *PA14_00180*, and *secA*.

Cross-Resistance and Collateral Susceptibility of the Evolved Populations

We have previously found that *P. aeruginosa* populations submitted to tobramycin or tigecycline present common changes in the susceptibility to other antibiotics besides those used along selection (Sanz-Garcia et al. 2018b). We wondered if the different phenotypic trajectories followed by *lasR** and PA14 populations could also result in differences in collateral susceptibility, reinforcing that the genetic background, either wild type or *lasR**, may constrain the evolutionary trajectories of *P. aeruginosa* in presence of antibiotics. To analyze this issue, the MICs of a set of antibiotics were determined for the 30 days populations that evolved from either PA14 or *lasR**.

In agreement with previous data (Sanz-Garcia et al. 2018a, 2018b), every population evolving in presence of antibiotics presented cross-resistance against antibiotics belonging to different structural families and were hyper-susceptible to fosfomycin (fig. 4 and supplementary table S6, Supplementary Material online). Fosfomycin hypersusceptibility has been associated with mutations in the genes that encode the *P. aeruginosa* peptidoglycan recycling pathway (Borisova et al. 2014; Hamou-Segarra et al. 2017), as well as with the inactivation of the fosfomycin resistance protein FosA (De Groote et al. 2011). Mutations in these genes were not found in the evolved populations. Nevertheless, it might be possible that their expression level could be altered in these populations, an issue that remains to be explored. Albeit, it is worth mentioning that quantitative differences in the strength of the phenotypic changes were observed between the two genetic backgrounds (fig. 4 and supplementary table S6, Supplementary Material online). *lasR**

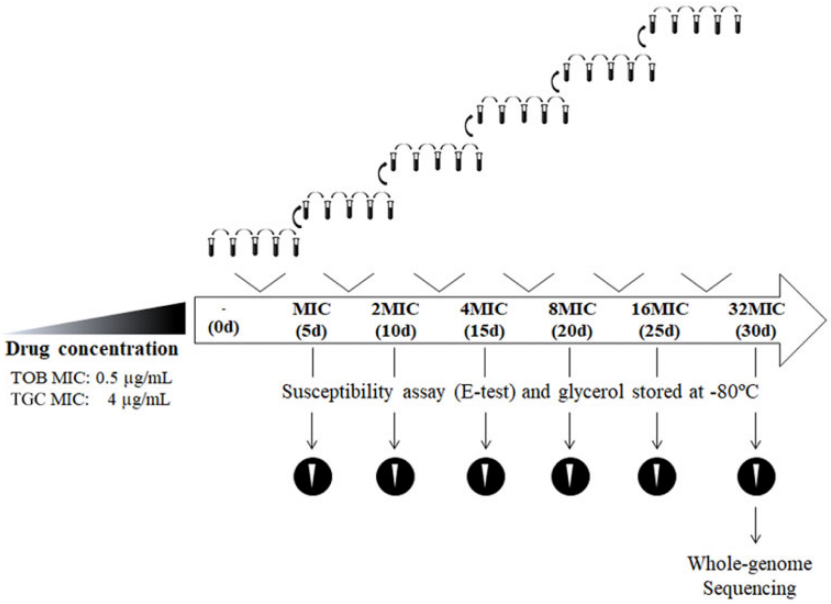

FIG. 1. Experimental evolution setup. Twelve independent bacterial populations of the *Pseudomonas aeruginosa lasR** mutant were grown in parallel in MH Broth (MHB) for 30 days. Four populations were challenged with tigecycline, four with tobramycin, and four were grown without any antibiotic. MICs of tigecycline and tobramycin were used to start the evolution experiment (0.5 µg/ml tobramycin; 4 µg/ml tigecycline). Every day cultures were diluted (1/250) in fresh MHB with the antibiotic concentration required. The concentration of the selection antibiotic was doubled every 5 days, increasing over the evolution experiment from the MIC up to 32MIC, and every replicate population from each step was preserved at −80 °C. MIC to the selection antibiotic for each replicate population was determined using *E*-test strips every 5 days. After 30 days of evolution, the genomic DNA of the 12 evolved populations was extracted and sequenced.

populations that had evolved in presence of tigecycline had higher ciprofloxacin and lower aminoglycosides (streptomycin and amikacin) MICs than PA14 populations evolved in presence of the same antibiotic. The tigecycline and tobramycin PA14 evolved populations showed a higher hypersusceptibility to fosfomycin than *lasR** populations that had evolved under the same conditions. Altogether, these results support the existence of epistatic interactions, which modulate the strength of the global phenotypes of susceptibility to antibiotics, between *lasR** and AR mutations. In a recent study of *P. aeruginosa* ALE, important differences in collateral susceptibility have been observed between parallel populations of the same genetic background adapted to the same antibiotic (Barbosa et al. 2017). These results and our findings suggest that historical contingency may be on the basis of the evolution of *P. aeruginosa* toward AR, including collateral susceptibility and cross-resistance phenotypic outcomes.

Analysis of Mutations Selected during *lasR** Experimental Evolution

To uncover the genetic modifications involved in the AR phenotype acquired by the populations of *lasR**, their genomes, as well as the genomes of the original *lasR** strain and of the control replicates, were sequenced at the end of the experiment (30 days), when the antibiotic concentration reached 32MIC. The mutations selected in the control populations were discarded for being considered to be involved in the adaptation to the growth medium. The presence of the mutations detected by WGS (supplementary table S2, Supplementary Material online) and the order of appearance of each mutation (supplementary table S3, Supplementary Material online) was confirmed by Sanger-sequencing of the corresponding genomic regions.

Population 5 presented, since the beginning of evolution, a mutation in *mutL*, a gene coding for a component of the mismatch repair system (Oliver et al. 2002), which inactivation renders a hypermutator phenotype characterized by an increased mutation rate and a particular mutational spectrum (mainly due to transitions) (Lee et al. 2012; Long et al. 2018). In agreement with this information, this population contains a larger number of mutations, most of them transitions, than the other ones (supplementary table S5, Supplementary Material online), not all of them necessarily involved in the acquisition of AR. Therefore, this population was not included in further comparative analysis.

The majority of the genetic changes found in the evolved populations resulted in amino acid alterations, frameshifts, or stop codons. Mutations in 20 different genes were candidates to be implicated in the acquisition of resistance to ribosome-targeting antibiotics in *P. aeruginosa lasR**. In agreement with results obtained when evolution was performed in PA14, these results indicate that increased resistance to tigecycline and tobramycin, two antibiotics targeting the same cellular machinery, comes about via distinguishable evolutionary trajectories, which have just two genes in common, *pmrB* and *orfN* (supplementary table S2, Supplementary Material online, and fig. 5). *pmrB* encodes the sensor of the two-component system PmrAB, involved in polymyxin and colistin resistance (Moskowitz et al. 2012; Jochumsen et al. 2016), whereas *orfN* encodes a putative glycosyl transferase needed for the

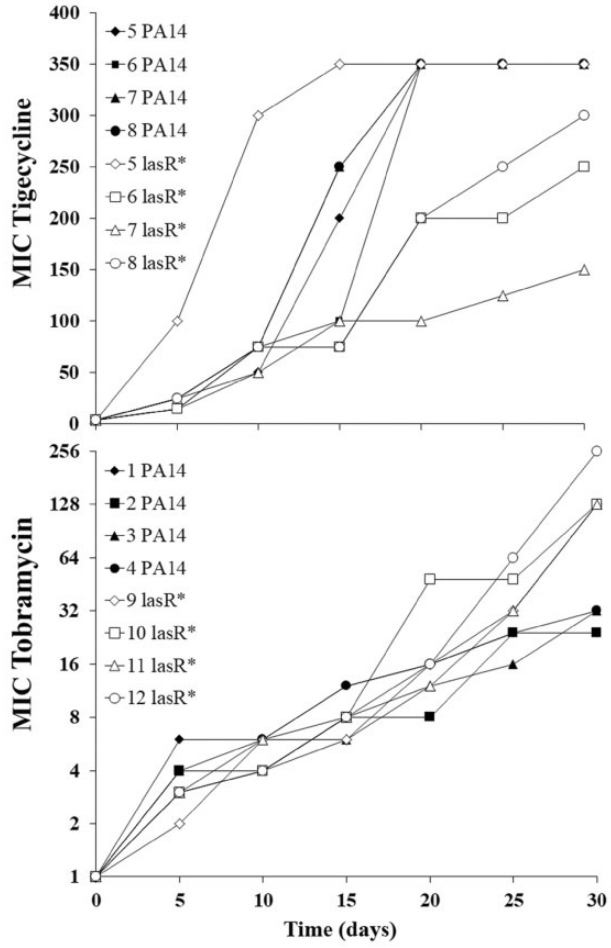

FIG. 2. Phenotypic evolutionary trajectories of the wild-type PA14 and *lasR** *Pseudomonas aeruginosa* population replicates evolving under antibiotic selective pressure. The figure shows the MIC values (μg/ml) for four PA14 and *lasR** replicates evolved in the presence of the indicated antibiotics. MIC values were determined using *E*-test strips in the case of tobramycin and a broth dilution method in the case of tigecycline, for which the detection limit of the *E*-test strip (256 μg/ml) was not high enough to determine the MICs.

glycosylation of type A flagellins (Schirm et al. 2004). Both genes have been described to mediate *P. aeruginosa* virulence and AR (Arora et al. 2005; Wong et al. 2012; Sanz-Garcia et al. 2018b). Interestingly, the populations lacking mutants in *orfN* contained mutations in *orfK* (9, 10 replicates) or in *orfH* (11 replicate), both genes belonging to the same genomic island implicated in flagellin glycosylation (Arora et al. 2004). In addition, it should be noted that these mutations are usually selected in early steps of the tigecycline evolution, suggesting that these genes may be important not only for bacterial virulence but also during the first stages of *P. aeruginosa* evolution toward resistance to the studied antibiotics.

The specific pathways toward resistance to each ribosome-targeting antibiotic were sought. In the case of tobramycin, mutations in *fusA* and *ptsP* were found in the first steps of all the replicates that evolved in presence of tobramycin (supplementary table S3, Supplementary Material online). *fusA* codes for an elongation factor whose mutations appear to be essential in the appearance of aminoglycosides resistance (Wang et al. 2015; Feng et al. 2016), whereas *ptsP* mutants have been selected under tobramycin pressure in a previous *P. aeruginosa* ALE study (Sanz-Garcia et al. 2018b).

Mutations in *nfxB*, encoding the transcriptional repressor of the multidrug efflux pump MexCD-OprJ (Purssell and Poole 2013), likely leading to *mexCD-OprJ* overexpression, were selected in all populations that had evolved in presence of tigecycline. An additional mutation in *mexD*, which codes for the transmembrane protein of the MexCD efflux pump, was detected in replicate 5 (supplementary table S5, Supplementary Material online). This mutation is located in one of the two large periplasmic loops involved in substrate specificity (Elkins and Nikaido 2002) and might improve MexD tigecycline specificity, a feature already described in the case of mutations in *acrB*, the *Enterobacteriaceae* orthologue of *mexD* (Blair et al. 2015). In addition of developing resistance to tigecycline, all the tigecycline evolved replicates showed increased resistance to antibiotics belonging to other structural categories such as quinolones, some beta-lactams and chloramphenicol (fig. 4 and supplementary table S6, Supplementary Material online), a set of antimicrobials that are MexCD substrates (De Kievit et al. 2001).

Comparison of Genetic Modifications Selected along *lasR** and PA14 Tobramycin or Tigecycline ALEs

As above described, the phenotypic evolutions of *lasR** and PA14 toward AR were different (fig. 3), which should be due to different genotypic trajectories followed by *P. aeruginosa* when submitted to antibiotic challenge in the two genomic backgrounds. Although a common set of mutated genes was selected in both cases, new mutations were selected in *lasR** challenged with either of the antibiotics (fig. 5). Moreover, some mutations selected in the wild-type strain PA14 were not selected in *lasR**. These results show that, besides affecting phenotypic evolutionary trajectories, the genetic background (either wild type or *lasR**) impacts as well the genotypic evolutionary trajectories of *P. aeruginosa* when submitted to selective pressure by either tobramycin or tigecycline.

In presence of tigecycline, mutations in *parRS*, *secAG*, and *PA14_00180*, which were selected during PA14 evolution (Sanz-Garcia et al. 2018b), were not selected in the *lasR** background, whereas new mutations were selected, in such *lasR** background, in *phoQ*, *rpoA*, and *mutL* (fig. 5). Population 5, which presents a *mutL* mutation, harbors also a larger number of mutations (supplementary table S2, Supplementary Material online). However, since this is a hypermutator population, these mutants were not included in further analysis. PhoP-PhoQ is a two-component regulatory system implicated in resistance to the polymyxin B and to aminoglycosides (Macfarlane et al. 2000). In addition, this two-component regulatory system regulates the expression of an ABC transporter system (PA4456-4452) which effluxes tetracycline (Chen and Duan 2016). *rpoA* encodes the RNA polymerase alpha subunit. It has been described that exposure to biocides selects *Salmonella enterica* serovar Typhimurium *rpoA* mutants presenting decreased

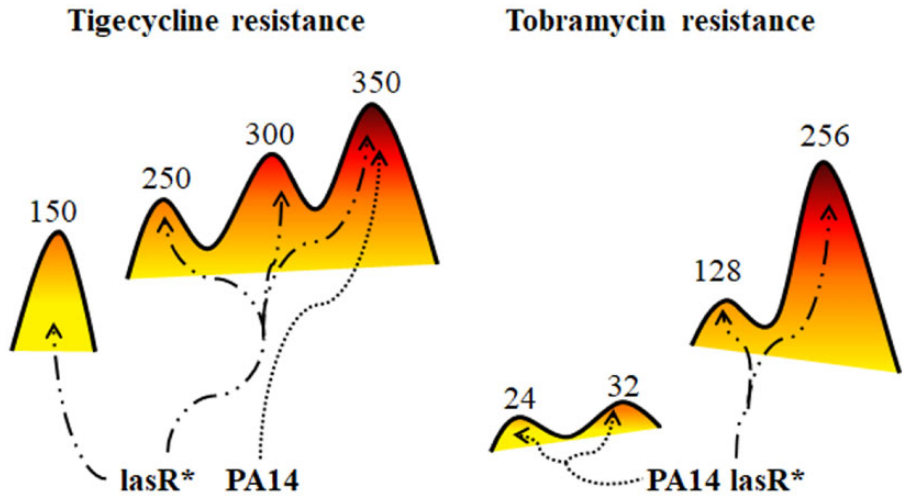

FIG. 3. *lasR** genotype constrains the evolutionary pathways of *Pseudomonas aeruginosa* toward AR. The evolutionary landscapes have been modeled as mountains with genotypes starting at the *X* axis and MIC values at the *Y* axis. High and low levels of resistance are represented as mountains with different altitude and levels of resistance inside each mountain are represented as mountain summits. The potential evolutionary trajectories followed by each genotype are represented.

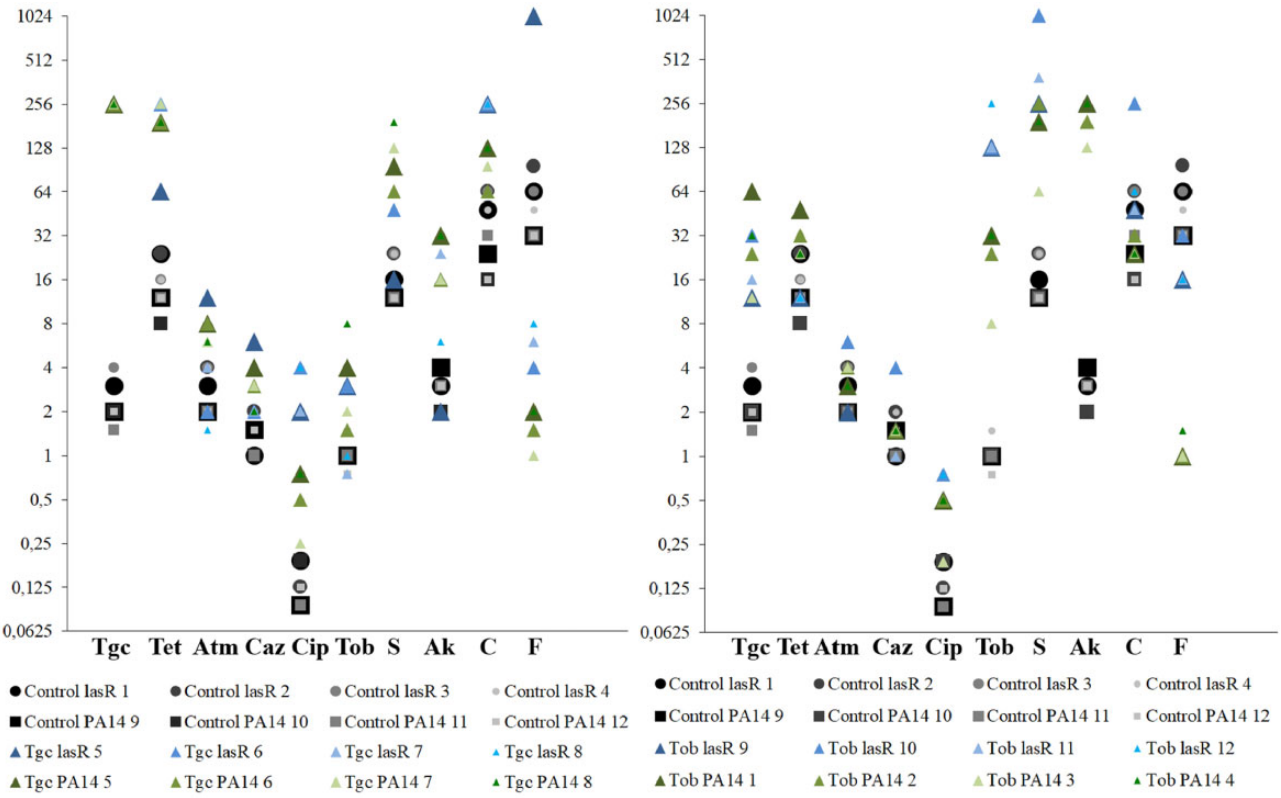

FIG. 4. Evolution of collateral sensitivity and cross-resistance. The collateral profiles of all experimentally evolved populations in tigecycline (left) and tobramycin (right) are represented. The antibiotics against which these populations were challenged are indicated in the *X* axis and the level of resistance for each individual replicate is indicated by a MIC value (μg/ml) in the *Y* axis. Squares and circles represent control replicates of *lasR** and PA14, respectively. Triangles represent each antibiotic treated replicate (different size for each replicate), being blue *lasR** and green PA14. Tgc, tigecycline; Tet, tetracycline; Atm, aztreonam; Caz, ceftazidime; Cip, ciprofloxacin; Tob, tobramycin; S, streptomycin; Ak, amikacin; C, chloramphenicol; and F, fosfomycin.

susceptibility to quinolones (Webber et al. 2015). Additionally, it has been reported that mutations in this gene are selected to compensate fitness defects of rifampicin resistant *Mycobacterium tuberculosis* isolates carrying *rpoB* mutations (Li et al. 2016; Nusrath Unissa and Hanna 2017). Whether *rpoA* mutations contribute to AR or they were selected as a compensation mechanism in our experiments remains to be established. As stated above, the mutation of

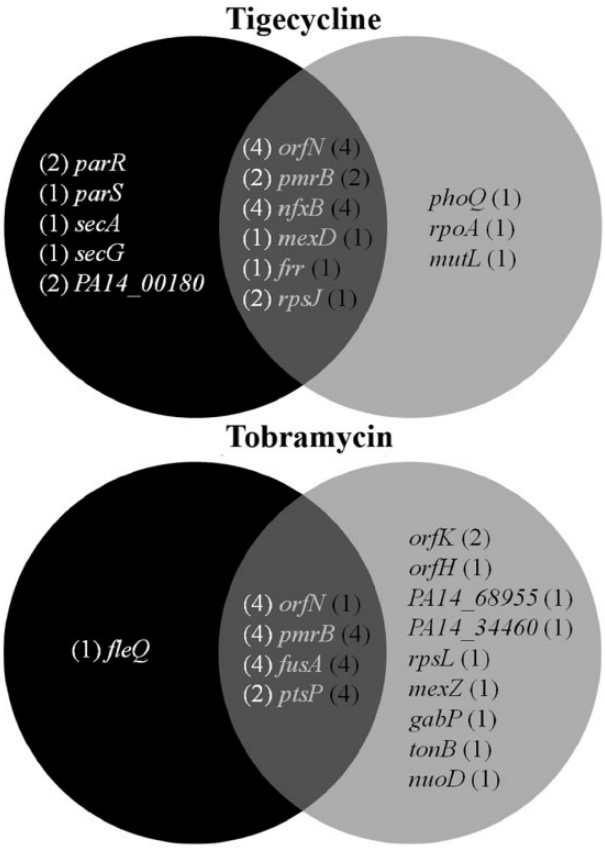

FIG. 5. Common and different genetic changes identified in *lasR** and PA14 populations of *Pseudomonas aeruginosa* evolved for 30 days in tigecycline and tobramycin. The graph shows the genes in which mutations were selected after evolution in 32MIC of antibiotics in *lasR** (gray) and wild-type PA14 (black) *P. aeruginosa* genomic backgrounds (Sanz-Garcia et al. 2018b). In parentheses, the number of replicates containing mutations in each gene is given.

mutL produces a hypermutator phenotype. This phenotype may allow a faster adaptation to antibiotic stress as described in the case of *P. aeruginosa* isolates causing chronic infections in CF patients (Oliver et al. 2002). It has been suggested that the inactivation of the DNA repair system may favor the selection of *lasR* mutations along *P. aeruginosa* chronic infections (Smania et al. 2004). However, other authors have described that mutations in *lasR* arise before mutations in the DNA repair system, showing that hyper-mutability is not a requisite for the acquisition of loss-of-function QS mutants (Ciofu et al. 2010). The results obtained in the current work are in agreement with this second possibility.

In the case of evolution in presence of tobramycin, loss-of-function of *lasR* gave rise to the selection of a set of new mutations, not previously selected in a wild-type PA14 background (Sanz-Garcia et al. 2018b), in *orfH*, *orfK*, *gabP*, *rpsL*, *tonB*, *nuoD*, *PA14_68955*, *PA14_34460*, and *mexZ*. The role played by *gabP*, *PA14_68955*, and *PA14_34460* in AR has not been previously described. *PA14_68955* and *PA14_34460* code for a 2-octaprenyl-3-methyl-6-methoxy-1, 4-benzoquinol hydroxylase and a hypothetical protein, respectively. *gabP* encodes a γ-aminobutyric acid permease. Its orthologous contributes to the growth and virulence of *P. syringae* in tomato plants (McCraw et al. 2016), but a role in AR of this gene has not been reported. The implication of *rpsL* mutations in resistance to ribosome-targeting antibiotics as streptomycin has been previously described in *Escherichia coli* (Pelchovich, Schreiber, et al. 2013) and *Mycobacterium tuberculosis* (Pelchovich, Zhuravlev, et al. 2013). *tonB* codes for an energy-transducing protein that couples the energy of the cytoplasmic membrane to a variety of outer membrane receptors required for the import of siderophores (Poole et al. 1996; Zhao and Poole 2002; Shirley and Lamont 2009). In addition, some *tonB* mutants are hyper-susceptible to antibiotics because of the involvement of TonB in the activity of some efflux pumps, such as MexAB (Zhao and Poole 2002). However, the nucleotide change selected in *tonB* in *lasR** replicate 10 did not produce any amino acid change, and this population was not susceptible to substrates typically extruded by MexAB, such as beta-lactams (Morita et al. 2001). *nuoD* is an already described tobramycin resistance determinant (Schurek et al. 2008). It encodes a proton-translocating NADH ubiquinone oxidoreductase that catalyzes the oxidation of NADH by ubiquinone. Mutations in *nuoD* lead to resistance to benzimidazole derivatives in *Helicobacter pylori* (Mills et al. 2004). It is possible that the Gln184* *nuoD* mutation causes a disruption of the electron transport chain (and the proton motive force), blocking

tobramycin uptake and increasing resistance. The Val43Gly substitution in MexZ, a transcriptional repressor of the *mexXY* multidrug efflux pump operon (Matsuo et al. 2004), should increase the expression of MexXY. Consistent with this situation, the Val43Gly *mexZ* mutation is associated with a reduced susceptibility to ciprofloxacin and tobramycin, two well-known MexXY substrates (Morita et al. 2001).

Genetic Analysis of Epistatic Interactions between *lasR** and Endpoint AR Mutations

Despite the mutations selected in both genetic backgrounds (wild type and *lasR**) present some degree of overlapping (fig. 5), the evolutionary trajectories were not the same, neither at the phenotypic (fig. 3) nor at the genotypic (fig. 5) levels. Further, although the *lasR** mutation was selected at early stages in the evolution of all the control PA14 populations, this mutation was not observed in any of the populations evolved in presence of antibiotic, neither at the first stages (5 days) nor at the end of the evolution (30 days). As shown in table 1, this is not the consequence of a reduced MIC on the *lasR** mutant to the antibiotic selective agents. A suitable hypothesis for explaining the observed counterselection of *lasR** mutants in the presence of antibiotics would be that the *lasR** allele reduces the fitness under selection (measured as growth in the presence of antibiotics) of the selected AR mutants. In other words, counterselection of *lasR** mutation in presence of antibiotics may be due to the existence of a negative epistasic interaction between *lasR** and some AR mutations (fig. 6).

To test this hypothesis, we isolated two resistant clones, containing a representative set of endpoint mutations, obtained at the end of PA14 evolution in presence of either tobramycin or tigecycline. For this purpose, ten independent clones were selected from each endpoint evolved population, the mutations present in the populations were confirmed by Sanger-sequencing. Representative clones containing a large number of mutations were chosen for further studies and their genomes were sequenced to ascertain that no other mutation was present. The clone obtained from the population PA14-2, which had evolved in presence of tobramycin, contained the following mutations: OrfN (Val50fs), FusA (Ala595Pro), PtsP (Glu677ST), FleQ (Thr241Pro), and PmrB (Ser8Ala). The clone obtained from the population PA14-5, which had evolved in presence of tigecycline, contained the mutations NfxB (Leu151Pro), OrfN (Val50fs), MexD (Phe608Cys), RpsJ (Val57Leu), ParR (Glu87Lys), PA14_00180 (Arg49Leu), and SecA (Ala492Val).

The *lasR** allele was introduced in both clones, and the genomes of the resulting strains were sequenced to ascertain that no other mutation was present. The effect of this mutation in the susceptibility to antibiotics was analyzed. As shown in table 1, the introduction of the *lasR** allele in the tobramycin resistant clone PA14-2 led to an increased susceptibility to this antibiotic, which was the one used for *P. aeruginosa* experimental evolution. This result supports the hypothesis that *lasR** mutants are counterselected in presence of this antibiotic as the consequence of negative epistasis with some AR mutations (fig. 6). Additionally, the introduction of the *lasR** allele led to changes in the cross-resistance phenotypes of clone PA14-2. In the *lasR** background, the mutant presents a higher susceptibility to amikacin and a decreased susceptibility to tigecycline and streptomycin (table 1) when compared with the wild-type PA14 background. These results further support the existence of epistasis between certain AR mutations, present in clone PA14-2, and *lasR**. In the case of the resistant clone PA14-5, isolated in a population that had evolved in presence of tigecycline, resistance to this antibiotic of both strains (PA14 or *lasR** background) was above the limit of detection of the test used for MIC determination. However, when bacteria grow in presence of this antibiotic, a clear impairment in growth rate and in the final optical density reached is found in the *lasR** genomic background, when compared with the original PA14-derived mutant (fig. 7). Besides, the introduction of the *lasR** allele modified the susceptibility to other antibiotics. In comparison with the situation in PA14, a *lasR** background increased the susceptibility to tobramycin, streptomycin and amikacin, and decreased the susceptibility to ciprofloxacin and fosfomycin of the tigecycline resistant clone PA14-5 (table 1). Again, these data support the existence of negative epistasis between *lasR** and certain AR mutations present in the tigecycline resistant clone PA14-5.

Epistatic Interactions between *lasR** and AR Mutations Occur at Early Stages of Evolution in Presence of Antibiotics

Our work indicates that the genomic background, either wild-type PA14 or QS-defective *lasR** mutant, modifies the strength of resistance as well as the collateral sensitivity and cross-resistance phenotypes of endpoint mutants selected in presence of either tobramycin or tygecycline. However, these results do not provide evidences on early stages of evolution, which would have been contingent for the establishment of the observed differential evolutionary trajectories. To address this issue, we focused in a mutation present in *orfN* (OrfNVal50fs), hereafter dubbed as *orfN**. This mutation was selected, at early stages of evolution (day 5), in all the *P. aeruginosa* PA14 populations evolved in the presence of either tigecycline or tobramycin (Sanz-Garcia et al. 2018b), so it may be contingent on PA14 evolution in presence of both antibiotics. As in the case of the wild-type PA14 background, *orfN** mutants were selected early in all *lasR** populations evolving in presence of tigecycline and in one of the populations evolving under tobramycin selection. These results indicate that, even in a *lasR** background, *orfN** mutations are frequently selected. *lasR** mutants are enriched in the first steps of evolution in absence of antibiotics, likely because they present an increased fitness on rich medium (supplementary fig. S3, Supplementary Material online). Therefore, it would be expected that, when fitter *lasR** mutants emerge and are mixed with the less fit PA14 population, *orfN** mutants should be more frequently selected in the *lasR** background than in the wild-type PA14 one. However, as above stated, the *lasR*orfN** mutant was not selected during evolution in presence of antibiotics, suggesting the existence of negative

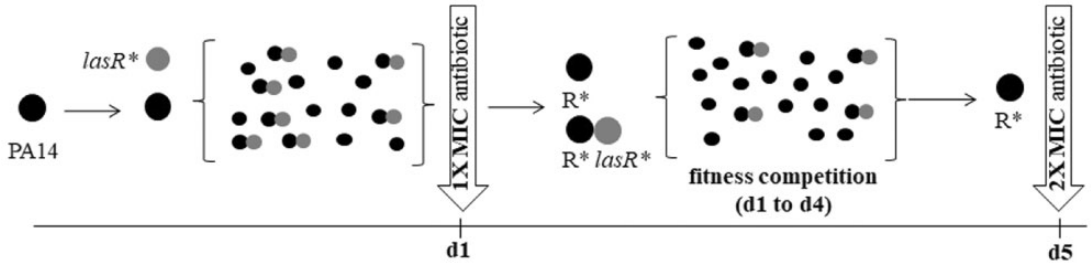

FIG. 6. Negative epistasis hypothesis between *lasR** and AR mutations. During growth in medium without antibiotic, the starting drug susceptible genotype (black circle) may present a mutation in *lasR* gene (gray circle) before the antibiotic treatment at day 1, this mutation is selected in all the control populations evolved in the absence of antibiotic. When antibiotic is applied, an AR mutation (R*) may be selected in both, PA14 and *lasR**. During maintenance of antibiotic concentration (day 1 to day 4) fitness competition may lead to a reduction of *lasR** mutants from day 1 to 4, being absent at day 5 and at the end of the evolutionary assay (see text for details).

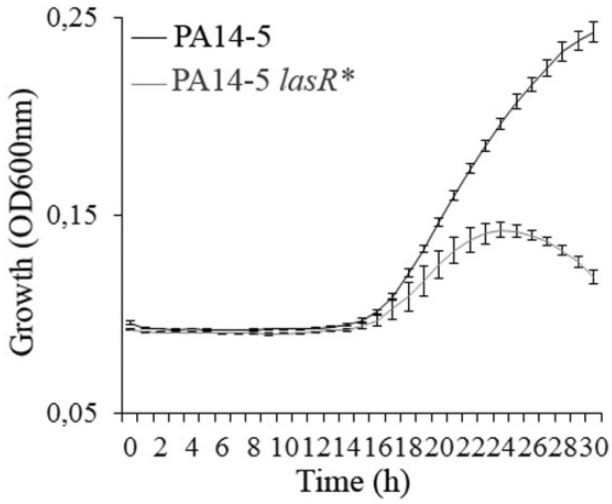

FIG. 7. Introduction of *lasR** in antibiotic resistant PA14 endpoint evolved clone reduces *Pseudomonas aeruginosa* fitness in presence of tigecycline. Growth curves on MHB containing tigecycline (50 μg/ml) of *P. aeruginosa* resistant mutant PA14-5 (*nfxB*, *orfN*, *mexD*, *rpsJ*, *parR*, *PA14_00180*, and *secA*) which contains (gray line) or not (black line) a *lasR** loss-of-function mutation. Mean ± SEM values of three replicates are represented.

epistatic interactions between *lasR** and *orfN** mutations. To address this possibility, single-step tigecycline resistant mutants were selected in plates containing tigecycline 32 μg/ml. *orfN* was Sanger-sequenced in 100 of the selected mutants. One *orfN** mutant was chosen for further studies and its genome was whole sequenced. The mutant presents the *orfN** mutation (OrfNVal50fs), without any further genetic modification. Since flagellum is relevant in *P. aeruginosa* motility (Overhage et al. 2007), it is possible that this mutation may alter such motility. Indeed, as shown in supplementary figure S4, Supplementary Material online, the *orfN** mutant presents a strongly reduced swarming motility (supplementary fig. S4, Supplementary Material online).

To analyze the effect of the genomic background in the level of resistance achieved by the *orfN** mutation, the *lasR** mutation was introduced into the *orfN** mutant. The genome of the resulting double mutant was whole sequenced, not presenting any further mutation. Growth curves of *orfN** and *lasR*orfN** mutants, in the presence of tobramycin or tigecycline, at the concentrations used in the previous evolution assay in PA14, were compared. As shown in figure 8, the introduction of the *lasR** mutation into the *orfN** mutant negatively affects the growth of *P. aeruginosa* in presence of either tobramycin or tigecycline, by reducting the final optical density reached and by increasing the lag phase, respectively. These results indicate that, when the *orfN** and *orfN*lasR** mutants coexist, the first should prevail over the second. In other words, the absence of *lasR** mutants in *P. aeruginosa* PA14 populations evolved in presence of either tobramycin or tigecycline was the consequence of negative epistasis between *lasR** and AR mutants, as *orfN**, selected early along evolution.

Conclusions

Although evolution is considered as largely unpredictable (Gould 1994), important efforts have been made to predict the evolution of microorganisms, particularly in the case of health relevant processes as the acquisition of AR (Martinez et al. 2011; Palmer and Kishony 2013; Hughes and Andersson 2017). It has been invoked that selective pressure, fitness costs, mutational load, and epistatic interactions modulate all of them the evolutionary trajectories of bacterial pathogens toward AR (Hughes and Andersson 2017). In addition, the order of acquisition of AR mutations—the historical contingency—is also in the basis of the evolutionary trajectories toward AR (Salverda et al. 2011). Herein, we analyze another aspect that may modulate the pathways toward AR, as is the existence of mutations in *loci* unrelated to the acquisition of resistance, in particular, those improving the fitness of the microorganisms when confronted with a new habitat (as the infected host). For this purpose, we studied the effect on *P. aeruginosa* evolution toward AR of a *lasR** loss-of-function mutation that improves bacterial fitness in absence of antibiotics, while impairing the QS response.

lasR mutations are frequently selected in vivo in *P. aeruginosa* populations causing chronic infections. It has been suggested that these mutations modify bacterial virulence, hence favoring chronic infections (Hoffman et al. 2009; Wilder et al. 2009; LaFayette et al. 2015). However, their selection in laboratory growing conditions suggests that optimization in the

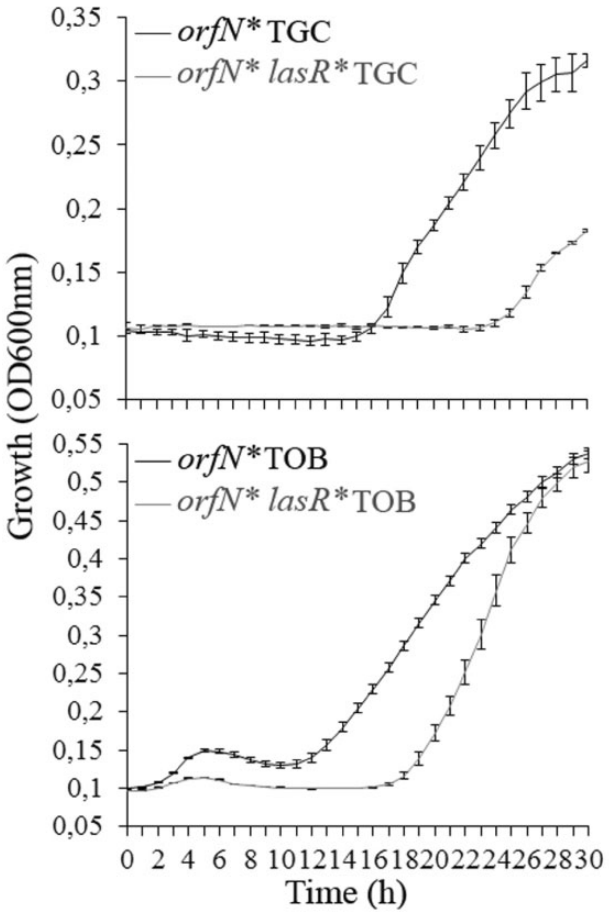

FIG. 8. Validation of the negative epistasis hypothesis between *lasR** loss-of-function and *orfN** mutations. Growth curves on MHB containing either tobramycin (0.5 μg/ml) or tigecycline (4 μg/ml) of *Pseudomonas aeruginosa orfN** mutant containing (gray line) or not (black line) a *lasR** mutation. Mean ± SEM values of three replicates are represented.

use of nutrients resources, more than fine tuning of bacterial/host interactions, is on the basis of the selection of these mutants. The current work indicates that negative epistasis and historical contingency modulate *P. aeruginosa* evolution toward resistance.

When *lasR** mutations are selected and predate the bacterial population early during evolution, before selection pressure is applied, AR mutants are selected in this genetic background similarly as it happens in a wild-type background. Nevertheless, *lasR** constrains the type and AR level of resistant mutants and therefore, competition between antibiotic resistant mutants, presenting either QS defective or QS wild-type backgrounds, is expected to happen. In turn, this indicates that the cross-talk between AR and virulence (Martinez and Baquero 2002) may depend on epistatic interactions among resistance and virulence determinants and that the trajectories leading to AR can be contingent on previously selected eco-adaptive mutations. Conversely, since QS-defective *lasR* mutants are not selected when AR mutants arise, QS-defective mutants are also contingent on previous acquisition of AR, at least in our experimental setting.

Materials and Methods

Experimental Evolution and Determination of Susceptibility to Antibiotics

Twelve independent *lasR** bacterial populations (four populations grown in presence of tigecycline, four populations challenged with tobramycin, and four control populations grown without antibiotic) were subjected to experimental evolution under the same conditions described in Sanz-Garcia et al. (2018b).

Cultures were grown at 37 °C and 250 rpm. Every day, along 30 days, the cultures were diluted (1/250) in fresh Mueller Hinton (MH) Broth (Pronadisa). Every 5 days the concentration of the selecting antibiotic was doubled, increasing over the evolution experiment from the MIC up to 32MIC. Every replicate population from each concentration step was preserved at −80 °C. In addition, every 5 days, the MIC of the antibiotic used for selection in population was determined at 37 °C in MH agar using *E*-test strips (MIC Test Strip, Liofilchem). A broth dilution method was performed in the case of tigecycline, for which the detection limit of the *E*-test strip (256 μg/ml) was not enough to evaluate the resistance level acquired by the evolved populations.

At the end of the experiment, the level of resistance acquired by the evolved populations and the susceptibility of these populations to a wide range of antibiotics—tetracycline, aztreonam, ceftazidime, ciprofloxacin, streptomycin, amikacin, chloramphenicol, and fosfomycin—was examined at 37 °C in MH agar using *E*-test strips.

WGS and Genomic Analysis

The genomic DNA of the 12 populations evolved during 30 days and of the mutants constructed in this work (*lasR**, *orfN**, *orfN*lasR**, *PA14-2*, *PA14-2 lasR**, *PA14-5*, and *PA14-5 lasR**) was extracted using the Gnome DNA kit (MP Biomedicals). WGS was performed by Sistemas Genómicos S.L. The quality analysis was done using a 4200 Tapestation High Sensitivity assay and the DNA concentration was determined by real-time polymerase chain reaction (PCR) using a LightCycler 480 device (Roche). Libraries constructed were pair-end (2× 100) and sequenced in an Illumina 2500 sequencer. The average number of reads per sample represents a coverage >200×. A similar approach was followed for sequencing the mutants used in the study.

WGS data were analyzed using the CLC Genomics Workbench 9.0 (QIAGEN) software. To that end, genomic information was trimmed and the reads were aligned against the *P. aeruginosa* UCBPP-PA14 reference chromosome (NC_008463.1). Mutations detected in the DNA samples obtained from populations kept under selective pressure were filtered against those mutations present in control populations.

Validation of Single Nucleotide Polymorphisms

Presence and order of appearance of the mutations detected in the WGS analysis was determined by Sanger-sequencing. Twenty pairs of primers, which amplified around 200 base pair regions containing each genetic modification, were used

(supplementary table S4, Supplementary Material online) to recover de DNA region by PCR. The DNA fragments were purified using the QIAquick PCR Purification Kit (QIAGEN) and Sanger-sequenced at GATC Biotech.

RNA Preparation and Real-Time Reverse Transcription-PCR

An overnight culture of *P. aeruginosa* PA14 was used to inoculate Erlenmeyer flasks with 20 ml of Lysogeny Broth (LB) Lennox medium to a final $OD_{600\,nm}$ of 0.01. The flasks were incubated until stationary phase of growth ($OD_{600\,nm} = 2, 5$). Ten milliliters of each culture were spun down at 4 °C (7,000 rpm) and frozen in dry ice. Total RNA was extracted with the Rneasy Kit (Qiagen). DNA was removed by treatment with the DNA-Free Kit (Ambion). To check that no residual DNA was present in the RNA samples, PCRs were performed with primers *rplU_Fw* and *rplU_Rv* (supplementary table S1, Supplementary Material online). cDNAs were obtained using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as indicated by the manufacturer.

Real-time reverse transcription-PCR was performed using a Power SYBR Green Kit (Applied Biosystems) in an ABI Prism 7300 real-time system (Applied Biosystems). The expression level of QS-related genes was measured using the primers described in supplementary table S1, Supplementary Material online. Gene expression data were normalized to that of the *rpsL* gene. All the primers used were designed with Primer3 Input software; their specificity was tested by BLAST alignment against *P. aeruginosa* PA14 genome from Pseudomonas Genome Database (http://www.pseudomonas.com/) and their efficiency was analyzed by reverse transcription-PCR using serial dilutions of cDNA. The relative amount of mRNA was calculated following the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). In all cases, the mean values of relative mRNA expression obtained in three independent triplicate experiments were considered.

Motility and Biofilm Assays

Swarming assays were carried out as described (Ha et al. 2014). The culture medium used contained 0.5% (w/v) Bacto Casaminoacids (BD), 3-mM $MgSO_4$, 3.3-mM K_2HPO_4, 0.5% (w/v) glucose, and 0.5% Bacto Agar (BD). Four microliters of bacterial overnight cultures diluted to an $OD_{600\,nm}$ of 1.0 were used to inoculate the plates.

Biofilm production was quantified using a microtiter plate assay (Merritt et al. 2005) with some minor modifications, using LB as culture medium, 0.1% (w/v) crystal violet in ethanol as staining solution and 0.25% Triton X-100 in ethanol as biofilm solvent solution. After 48 h of culture in static conditions at 37 °C, the $OD_{570\,nm}$ of stained, washed, and solubilized contents of each well, were measured using a Tecan Infinite 200 plate reader (TECAN).

Growth Curves

Growth curves were determined using a Tecan Infinite 200 plate reader (TECAN) by measuring the $OD_{600\,nm}$ of the bacterial cultures (inoculated at an $OD_{600\,nm}$ of 0.01) every 10 min during 30 h at 37 °C in MH media and MH media supplemented with different concentrations of tobramycin and tigecycline.

Mutant Construction

Strains containing the *lasR** selected mutation were constructed by insertion of the mutant allele by homologous recombination. *lasR** allele was obtained by PCR from the mutant strain obtained in the evolutionary assay, leaving ~500 bp upstream and downstream of the corresponding single nucleotide polymorphism using the oligonucleotides 5′-*AAGCTT*AGCGCCATCCTGCAGAAGAT-3′ and 5′-*AAGCTT*GCCGACCAATTTGTACGATC-3. The PCR product containing *Hin*dIII restriction sites was cloned into *Hin*dIII-digested and dephosphorylated pEX18Ap vector (Hoang et al. 1998), and then introduced by transformation into the conjugative *Escherichia coli* S17-1 strain. Subsequently, conjugation and mutant selection were performed as described elsewhere (Hoang et al. 1998) using 350 µg/ml carbenicillin and 10% sucrose.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

Acknowledgments

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References

Agnello M, Finkel SE, Wong-Beringer A. 2016. Fitness cost of fluoroquinolone resistance in clinical isolates of *Pseudomonas aeruginosa* differs by type III secretion genotype. *Front Microbiol*. 7:1591.

Andersson DI, Hughes D. 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol*. 8(4):260–271.

Arora SK, Neely AN, Blair B, Lory S, Ramphal R. 2005. Role of motility and flagellin glycosylation in the pathogenesis of *Pseudomonas aeruginosa* burn wound infections. *Infect Immun*. 73(7):4395–4398.

Arora SK, Wolfgang MC, Lory S, Ramphal R. 2004. Sequence polymorphism in the glycosylation island and flagellins of *Pseudomonas aeruginosa*. *J Bacteriol*. 186(7):2115–2122.

Baquero F. 2013. Epigenetics, epistasis and epidemics. *Evol Med Public Health* 2013(1):86–88.

Baquero F, Alvarez-Ortega C, Martinez JL. 2009. Ecology and evolution of antibiotic resistance. *Environ Microbiol Rep*. 1(6):469–476.

Baquero F, Martinez JL. 2017. Interventions on Metabolism: Making Antibiotic-Susceptible Bacteria. *MBio* 8(6): pii: e01950-17.

Barbosa C, Trebosc V, Kemmer C, Rosenstiel P, Beardmore R, Schulenburg H, Jansen G. 2017. Alternative evolutionary paths to bacterial antibiotic resistance cause distinct collateral effects. *Mol Biol Evol*. 34(9):2229–2244.

Barrick JE, Yu DS, Yoon SH, Jeong H, Oh TK, Schneider D, Lenski RE, Kim JF. 2009. Genome evolution and adaptation in a long-term experiment with *Escherichia coli*. *Nature* 461(7268):1243–1247.

Blair JMA, Bavro VN, Ricci V, Modi N, Cacciotto P, Kleinekathöfer U, Ruggerone P, Vargiu AV, Baylay AJ, Smith HE. 2015. AcrB drug-

binding pocket substitution confers clinically relevant resistance and altered substrate specificity. *Proc Natl Acad Sci U S A*. 112(11):3511–3516.
Blount ZD, Lenski RE, Losos JB. 2018. Contingency and determinism in evolution: replaying life's tape. *Science* 362(6415): pii: eaam5979.
Borisova M, Gisin J, Mayer C. 2014. Blocking peptidoglycan recycling in *Pseudomonas aeruginosa* attenuates intrinsic resistance to fosfomycin. *Microb Drug Resist*. 20(3):231–237.
Chen L, Duan K. 2016. A PhoPQ-regulated ABC transporter system exports tetracycline in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 60(5):3016–3024.
Ciofu O, Mandsberg LF, Bjarnsholt T, Wassermann T, Hoiby N. 2010. Genetic adaptation of *Pseudomonas aeruginosa* during chronic lung infection of patients with cystic fibrosis: strong and weak mutators with heterogeneous genetic backgrounds emerge in mucA and/or lasR mutants. *Microbiology (Reading, Engl)*. 156(Pt 4):1108–1119.
De Groote VN, Fauvart M, Kint CI, Verstraeten N, Jans A, Cornelis P, Michiels J. 2011. *Pseudomonas aeruginosa* fosfomycin resistance mechanisms affect non-inherited fluoroquinolone tolerance. *J Med Microbiol*. 60(Pt 3):329–336.
De Kievit TR, Parkins MD, Gillis RJ, Srikumar R, Ceri H, Poole K, Iglewski BH, Storey DG. 2001. Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother*. 45(6):1761–1770.
de Visser JA, Krug J. 2014. Empirical fitness landscapes and the predictability of evolution. *Nat Rev Genet*. 15(7):480–490.
Elkins CA, Nikaido H. 2002. Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of *Escherichia coli* is determined predominantly by two large periplasmic loops. *J Bacteriol*. 184(23):6490–6498.
Fajardo A, Linares JF, Martinez JL. 2009. Towards an ecological approach to antibiotics and antibiotic resistance genes. *Clin Microbiol Infect*. 15 (Suppl 1):14–16.
Feng Y, Jonker MJ, Moustakas I, Brul S, Ter Kuile BH. 2016. Dynamics of mutations during development of resistance by *Pseudomonas aeruginosa* against five antibiotics. *Antimicrob Agents Chemother*. 60(7):4229–4236.
Fuzi M. 2016. Dissimilar fitness associated with resistance to fluoroquinolones influences clonal dynamics of various multiresistant bacteria. *Front Microbiol*. 7: 1017.
Gifford DR, Toll-Riera M, MacLean RC. 2016. Epistatic interactions between ancestral genotype and beneficial mutations shape evolvability in *Pseudomonas aeruginosa*. *Evolution* 70(7):1659–1666.
Gould SJ. 1994. The evolution of life on the Earth. *Sci Am*. 271(4):84–91.
Ha DG, Kuchma SL, O'Toole GA. 2014. Plate-based assay for swarming motility in *Pseudomonas aeruginosa*. *Methods Mol Biol*. 1149:67–72.
Hamou-Segarra M, Zamorano L, Vadlamani G, Chu M, Sanchez-Diener I, Juan C, Blazquez J, Hattie M, Stubbs KA, Mark BL, et al. 2017. Synergistic activity of fosfomycin, beta-lactams and peptidoglycan recycling inhibition against *Pseudomonas aeruginosa*. *J Antimicrob Chemother*. 72(2):448–454.
Hernando-Amado S, Sanz-Garcia F, Blanco P, Martinez JL. 2017. Fitness costs associated with the acquisition of antibiotic resistance. *Essays Biochem*. 61(1):37–48.
Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212(1):77–86.
Hoffman LR, Kulasekara HD, Emerson J, Houston LS, Burns JL, Ramsey BW, Miller SI. 2009. *Pseudomonas aeruginosa lasR* mutants are associated with cystic fibrosis lung disease progression. *J Cyst Fibros*. 8(1):66–70.
Hughes D, Andersson DI. 2017. Evolutionary trajectories to antibiotic resistance. *Annu Rev Microbiol*. 71:579–596.
Huse HK, Kwon T, Zlosnik JE, Speert DP, Marcotte EM, Whiteley M. 2010. Parallel evolution in *Pseudomonas aeruginosa* over 39,000 generations in vivo. *MBio* 1(4):e00199-10.
Huseby DL, Pietsch F, Brandis G, Garoff L, Tegehall A, Hughes D. 2017. Mutation supply and relative fitness shape the genotypes of ciprofloxacin-resistant *Escherichia coli*. *Mol Biol Evol*. 34(5):1029–1039.
Ibacache-Quiroga C, Oliveros JC, Couce A, Blazquez J. 2018. Parallel evolution of high-level aminoglycoside resistance in *Escherichia coli* under low and high mutation supply rates. *Front Microbiol*. 9:427.
Imamovic L, Ellabaan MMH, Dantas Machado AM, Citterio L, Wulff T, Molin S, Krogh Johansen H, Sommer M. 2018. Drug-driven phenotypic convergence supports rational treatment strategies of chronic infections. *Cell* 172(1-2):121–134.e114.
Jochumsen N, Marvig RL, Damkiær S, Jensen RL, Paulander W, Molin S, Jelsbak L, Folkesson A. 2016. The evolution of antimicrobial peptide resistance in *Pseudomonas aeruginosa* is shaped by strong epistatic interactions. *Nat Commun*. 7:13002.
Kiratisin P, Tucker KD, Passador L. 2002. LasR, a transcriptional activator of *Pseudomonas aeruginosa* virulence genes, functions as a multimer. *J Bacteriol*. 184(17):4912–4919.
Knopp M, Andersson DI. 2018. Predictable phenotypes of antibiotic resistance mutations. *MBio* 9(3): pii: e00770-18.
Knoppel A, Nasvall J, Andersson DI. 2017. Evolution of antibiotic resistance without antibiotic exposure. *Antimicrob Agents Chemother*. 61(11):pii: e01495-17.
Kryazhimskiy S, Rice DP, Jerison ER, Desai MM. 2014. Microbial evolution. Global epistasis makes adaptation predictable despite sequence-level stochasticity. *Science* 344(6191):1519–1522.
LaFayette SL, Houle D, Beaudoin T, Wojewodka G, Radzioch D, Hoffman LR, Burns JL, Dandekar AA, Smalley NE, Chandler JR. 2015. Cystic fibrosis-adapted *Pseudomonas aeruginosa* quorum sensing lasR mutants cause hyperinflammatory responses. *Sci Adv*. 1(6):pii: e1500199.
Lazar V, Pal Singh G, Spohn R, Nagy I, Horvath B, Hrtyan M, Busa-Fekete R, Bogos B, Mehi O, Csorgo B, et al. 2013. Bacterial evolution of antibiotic hypersensitivity. *Mol Syst Biol*. 9:700.
Lee H, Popodi E, Tang H, Foster PL. 2012. Rate and molecular spectrum of spontaneous mutations in the bacterium *Escherichia coli* as determined by whole-genome sequencing. *Proc Natl Acad Sci U S A*. 109(41):E2774–E2783.
Levin BR, Rozen DE. 2006. Non-inherited antibiotic resistance. *Nat Rev Microbiol*. 4(7):556–562.
Li QJ, Jiao WW, Yin QQ, Xu F, Li JQ, Sun L, Xiao J, Li YJ, Mokrousov I, Huang HR, et al. 2016. Compensatory mutations of rifampin resistance are associated with transmission of multidrug-resistant *Mycobacterium tuberculosis* Beijing genotype strains in China. *Antimicrob Agents Chemother*. 60(5):2807–2812.
Linares JF, Lopez JA, Camafeita E, Albar JP, Rojo F, Martinez JL. 2005. Overexpression of the multidrug efflux pumps MexCD-OprJ and MexEF-OprN is associated with a reduction of type III secretion in *Pseudomonas aeruginosa*. *J Bacteriol*. 187(4):1384–1391.
Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-DDCT} method. *Methods*. 25(4):402–408.
Long H, Miller SF, Williams E, Lynch M. 2018. Specificity of the DNA mismatch repair system (MMR) and mutagenesis bias in bacteria. *Mol Biol Evol*. 35(10):2414–2421.
Macfarlane EL, Kwasnicka A, Hancock RE. 2000. Role of *Pseudomonas aeruginosa* PhoP-PhoQ in resistance to antimicrobial cationic peptides and aminoglycosides. *Microbiology* 146(10):2543–2554.
Maddamsetti R, Lenski RE, Barrick JE. 2015. Adaptation, clonal interference, and frequency-dependent interactions in a long-term evolution experiment with *Escherichia coli*. *Genetics* 200(2):619–631.
Martinez JL, Baquero F. 2002. Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance. *Clin Microbiol Rev*. 15(4):647–679.
Martinez JL, Baquero F, Andersson DI. 2011. Beyond serial passages: new methods for predicting the emergence of resistance to novel antibiotics. *Curr Opin Pharmacol*. 11(5):439–445.

Martinez JL, Fajardo A, Garmendia L, Hernandez A, Linares JF, Martinez-Solano L, Sanchez MB. 2009. A global view of antibiotic resistance. *FEMS Microbiol Rev*. 33(1):44–65.

Martinez JL, Rojo F. 2011. Metabolic regulation of antibiotic resistance. *FEMS Microbiol Rev*. 35(5):768–789.

Martinez-Solano L, Macia MD, Fajardo A, Oliver A, Martinez JL. 2008. Chronic *Pseudomonas aeruginosa* infection in chronic obstructive pulmonary disease. *Clin Infect Dis*. 47(12):1526–1533.

Matsuo Y, Eda S, Gotoh N, Yoshihara E, Nakae T. 2004. MexZ-mediated regulation of *mexXY* multidrug efflux pump expression in *Pseudomonas aeruginosa* by binding on the *mexZ-mexX* intergenic DNA. *FEMS Microbiol Lett*. 238(1):23–28.

McCraw SL, Park DH, Jones R, Bentley MA, Rico A, Ratcliffe RG, Kruger NJ, Collmer A, Preston GM. 2016. GABA (gamma-aminobutyric acid) uptake via the GABA permease GabP represses virulence gene expression in *Pseudomonas syringae* pv. *tomato* DC3000. *Mol Plant Microbe Interact*. 29(12):938–949.

Merritt JH, Kadouri DE, O'Toole GA. 2005. Growing and analyzing static biofilms. *Curr Protoc Microbiol*. Chapter 1:Unit 1B 1.

Mills SD, Yang W, MacCormack K. 2004. Molecular characterization of benzimidazole resistance in *Helicobacter pylori*. *Antimicrob Agents Chemother*. 48(7):2524–2530.

Morita Y, Kimura N, Mima T, Mizushima T, Tsuchiya T. 2001. Roles of MexXY- and MexAB-multidrug efflux pumps in intrinsic multidrug resistance of *Pseudomonas aeruginosa* PAO1. *J Gen Appl Microbiol*. 47(1):27–32.

Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, Selgrade SE, Miller SI, Denton M, Conway SP, et al. 2012. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother*. 56(2):1019–1030.

Nichol D, Rutter J, Bryant C, Hujer AM, Lek S, Adams MD, Jeavons P, Anderson ARA, Bonomo RA, Scott JG. 2019. Antibiotic collateral sensitivity is contingent on the repeatability of evolution. *Nat Commun*. 10(1):334.

Novais A, Comas I, Baquero F, Canton R, Coque TM, Moya A, Gonzalez-Candelas F, Galan JC. 2010. Evolutionary trajectories of beta-lactamase CTX-M-1 cluster enzymes: predicting antibiotic resistance. *PLoS Pathog*. 6(1):e1000735.

Nusrath Unissa A, Hanna LE. 2017. Molecular mechanisms of action, resistance, detection to the first-line anti tuberculosis drugs: rifampicin and pyrazinamide in the post whole genome sequencing era. *Tuberculosis (Edinb)*. 105:96–107.

Oliver A, Baquero F, Blazquez J. 2002. The mismatch repair system (*mutS*, *mutL* and *uvrD* genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. *Mol Microbiol*. 43(6):1641–1650.

Overhage J, Lewenza S, Marr AK, Hancock RE. 2007. Identification of genes involved in swarming motility using a *Pseudomonas aeruginosa* PAO1 *mini-Tn5-lux* mutant library. *J Bacteriol* 189:2164–2169.

Palmer AC, Kishony R. 2013. Understanding, predicting and manipulating the genotypic evolution of antibiotic resistance. *Nat Rev Genet*. 14(4):243–248.

Pelchovich G, Schreiber R, Zhuravlev A, Gophna U. 2013. The contribution of common *rpsL* mutations in *Escherichia coli* to sensitivity to ribosome targeting antibiotics. *Int J Med Microbiol*. 303(8):558–562.

Pelchovich G, Zhuravlev A, Gophna U. 2013. Effect of ribosome-targeting antibiotics on streptomycin-resistant *Mycobacterium* mutants in the *rpsL* gene. *Int J Antimicrob Agents* 42(2):129–132.

Poole K, Zhao Q, Neshat S, Heinrichs DE, Dean CR. 1996. The *Pseudomonas aeruginosa tonB* gene encodes a novel TonB protein. *Microbiology* 142(Pt 6):1449–1458.

Purssell A, Poole K. 2013. Functional characterization of the NfxB repressor of the *mexCD-oprJ* multidrug efflux operon of *Pseudomonas aeruginosa*. *Microbiology (Reading, Engl)*. 159(Pt 10):2058–2073.

Rodriguez-Verdugo A, Gaut BS, Tenaillon O. 2013. Evolution of *Escherichia coli* rifampicin resistance in an antibiotic-free environment during thermal stress. *BMC Evol Biol*. 13:50.

Rosenkilde CEH, Munck C, Porse A, Linkevicius M, Andersson DI, Sommer M. 2019. Collateral sensitivity constrains resistance evolution of the CTX-M-15 beta-lactamase. *Nat Commun*. 10(1):618.

Ruparell A, Dubern JF, Ortori CA, Harrison F, Halliday NM, Emtage A, Ashawesh MM, Laughton CA, Diggle SP, Williams P, et al. 2016. The fitness burden imposed by synthesising quorum sensing signals. *Sci Rep*. 6:33101.

Salverda ML, Dellus E, Gorter FA, Debets AJ, van der Oost J, Hoekstra RF, Tawfik DS, de Visser JA. 2011. Initial mutations direct alternative pathways of protein evolution. *PLoS Genet*. 7(3):e1001321.

Sander P, Springer B, Prammananan T, Sturmfels A, Kappler M, Pletschette M, Bottger EC. 2002. Fitness cost of chromosomal drug resistance-conferring mutations. *Antimicrob Agents Chemother*. 46(5):1204–1211.

Sandoz KM, Mitzimberg SM, Schuster M. 2007. Social cheating in *Pseudomonas aeruginosa* quorum sensing. *Proc Natl Acad Sci U S A*. 104(40):15876–15881.

Sanz-Garcia F, Hernando-Amado S, Martinez JL. 2018a. Mutation-driven evolution of *Pseudomonas aeruginosa* in the presence of either ceftazidime or ceftazidime-avibactam. *Antimicrob Agents Chemother*. 62(10): pii: e01379-18.

Sanz-Garcia F, Hernando-Amado S, Martinez JL. 2018b. Mutational evolution of *Pseudomonas aeruginosa* resistance to ribosome-targeting antibiotics. *Front Genet*. 9:451.

Schenk MF, de Visser JA. 2013. Predicting the evolution of antibiotic resistance. *BMC Biol*. 11:14.

Schenk MF, Szendro IG, Salverda ML, Krug J, de Visser JA. 2013. Patterns of epistasis between beneficial mutations in an antibiotic resistance gene. *Mol Biol Evol*. 30(8):1779–1787.

Schirm M, Arora SK, Verma A, Vinogradov E, Thibault P, Ramphal R, Logan SM. 2004. Structural and genetic characterization of glycosylation of type a flagellin in *Pseudomonas aeruginosa*. *J Bacteriol*. 186(9):2523–2531.

Schurek KN, Marr AK, Taylor PK, Wiegand I, Semenec L, Khaira BK, Hancock RE. 2008. Novel genetic determinants of low-level aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 52(12):4213–4219.

Shcherbakov D, Akbergenov R, Matt T, Sander P, Andersson DI, Bottger EC. 2010. Directed mutagenesis of *Mycobacterium smegmatis* 16S rRNA to reconstruct the in-vivo evolution of aminoglycoside resistance in *Mycobacterium tuberculosis*. *Mol Microbiol*. 77(4):830–840.

Shirley M, Lamont IL. 2009. Role of TonB1 in pyoverdine-mediated signaling in *Pseudomonas aeruginosa*. *J Bacteriol*. 191(18):5634–5640.

Skurnik D, Roux D, Cattoir V, Danilchanka O, Lu X, Yoder-Himes DR, Han K, Guillard T, Jiang D, Gaultier C, et al. 2013. Enhanced in vivo fitness of carbapenem-resistant *oprD* mutants of *Pseudomonas aeruginosa* revealed through high-throughput sequencing. *Proc Natl Acad Sci U S A*. 110(51):20747–20752.

Smania AM, Segura I, Pezza RJ, Becerra C, Albesa I, Argarana CE. 2004. Emergence of phenotypic variants upon mismatch repair disruption in *Pseudomonas aeruginosa*. *Microbiology (Reading, Engl)*. 150(Pt 5):1327–1338.

Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM, et al. 2006. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci U S A*. 103(22):8487–8492.

Szamecz B, Boross G, Kalapis D, Kovacs K, Fekete G, Farkas Z, Lazar V, Hrtyan M, Kemmeren P, Groot Koerkamp MJ, et al. 2014. The genomic landscape of compensatory evolution. *PLoS Biol*. 12(8):e1001935.

Tenaillon O, Rodriguez-Verdugo A, Gaut RL, McDonald P, Bennett AF, Long AD, Gaut BS. 2012. The molecular diversity of adaptive convergence. *Science* 335(6067):457–461.

Toprak E, Veres A, Michel JB, Chait R, Hartl DL, Kishony R. 2011. Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nat Genet*. 44(1):101–105.

Trindade S, Sousa A, Xavier KB, Dionisio F, Ferreira MG, Gordo I. 2009. Positive epistasis drives the acquisition of multidrug resistance. *PLoS Genet*. 5(7):e1000578.

Vogwill T, Kojadinovic M, MacLean RC. 2016. Epistasis between antibiotic resistance mutations and genetic background shape the fitness effect of resistance across species of *Pseudomonas*. *Proc Biol Sci*. 283(1830):pii: 20160151.

Wang D, Dorosky RJ, Han CS, Lo CC, Dichosa AE, Chain PS, Yu JM, Pierson LS 3rd, Pierson EA. 2015. Adaptation genomics of a small-colony variant in a *Pseudomonas chlororaphis* 30–84 biofilm. *Appl Environ Microbiol*. 81(3):890–899.

Ward H, Perron GG, Maclean RC. 2009. The cost of multiple drug resistance in *Pseudomonas aeruginosa*. *J Evol Biol*. 22(5):997–1003.

Webber MA, Whitehead RN, Mount M, Loman NJ, Pallen MJ, Piddock L. 2015. Parallel evolutionary pathways to antibiotic resistance selected by biocide exposure. *J Antimicrob Chemother*. 70(8):2241–2248.

Weinreich DM. 2005. The rank ordering of genotypic fitness values predicts genetic constraint on natural selection on landscapes lacking sign epistasis. *Genetics* 171(3):1397–1405.

Weinreich DM, Delaney NF, Depristo MA, Hartl DL. 2006. Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science* 312(5770):111–114.

Wilder CN, Allada G, Schuster M. 2009. Instantaneous within-patient diversity of *Pseudomonas aeruginosa* quorum-sensing populations from cystic fibrosis lung infections. *Infect Immun*. 77(12):5631–5639.

Williams P, Winzer K, Chan WC, Camara M. 2007. Look who's talking: communication and quorum sensing in the bacterial world. *Philos Trans R Soc Lond B Biol Sci*. 362(1483):1119–1134.

Wong A. 2017. Epistasis and the evolution of antimicrobial resistance. *Front Microbiol*. 8:246.

Wong A, Rodrigue N, Kassen R. 2012. Genomics of adaptation during experimental evolution of the opportunistic pathogen *Pseudomonas aeruginosa*. *PLoS Genet*. 8(9):e1002928.

Yang L, Jelsbak L, Marvig RL, Damkiaer S, Workman CT, Rau MH, Hansen SK, Folkesson A, Johansen HK, Ciofu O, et al. 2011. Evolutionary dynamics of bacteria in a human host environment. *Proc Natl Acad Sci U S A*. 108(18):7481–7486.

Zhao K, Zhou X, Li W, Zhang X, Yue B. 2016. Nutrient reduction induced stringent responses promote bacterial quorum-sensing divergence for population fitness. *Sci Rep*. 6:34925.

Zhao Q, Poole K. 2002. Differential effects of mutations in *tonB1* on intrinsic multidrug resistance and iron acquisition in *Pseudomonas aeruginosa*. *J Bacteriol*. 184(7):2045–2049.

Artículo IV

The evolutionary landscapes of *Pseudomonas aeruginosa* towards ribosome-targeting antibiotics resistance depend on the selection strength

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Durante varias décadas se ha asumido que los mutantes resistentes a antibióticos son seleccionados en un rango de concentraciones situado entre la CMI de la cepa silvestre y la CPM. No obstante, actualmente se sabe que esta ventana de selección incluye concentraciones subinhibitorias -inferiores a la CMI-, lo que acarrea una expansión de los ambientes y condiciones en los que la selección de resistencia puede tener lugar.

En este trabajo se ha efectuado una comparación entre los resultados de la antecitada ALE de *P. aeruginosa* PA14 en concentraciones letales crecientes de tobramicina y tigeciclina (Artículo II), y una nueva ALE realizada con idénticos parámetros, a excepción de la presión de selección, que se impuso en concentraciones subletales constantes durante la evolución: 1/10 y 1/50 de la CMI de la cepa silvestre para ambos antimicrobianos. En el caso de la tigeciclina se seleccionaron mutantes resistentes en ambas condiciones, algo que no sucedió con la tobramicina. Este primer dato sugiere que la ventana de selección subletal es específica de cada antibiótico.

Profundizando en la comparación de los resultados derivados de las ALEs en presencia de distintas concentraciones de tigeciclina, se observó que los patrones evolutivos genotípicos y fenotípicos dependían de la presión de selección. Concentraciones subletales de tigeciclina condujeron a la selección de mutantes con menores niveles de resistencia frente a esta glicilciclina, pero también a algunos casos más acusados de resistencia cruzada (especialmente para las quinolonas), en comparación a lo acontecido cuando las bacterias eran expuestas a concentraciones letales de la droga. Este hecho apunta a que la naturaleza del fenotipo de sensibilidad a antibióticos diferentes al usado en la selección no tiene por qué ir de la mano de la presión de dicha selección; que ésta sea menor no conlleva necesariamente un menor número o grado de resistencias cruzadas. Aparte, también se manifestaron diferencias en el genotipo, puesto que *orfN* fue el único gen en el que se seleccionaron mutaciones en ambas condiciones.

Así, a raíz de toda esta información, se torna necesario determinar adecuadamente la ventana de selección de cada antibiótico de interés clínico, a la par que el fenotipo de sensibilidad a otros agentes antimicrobianos que presenten los mutantes surgidos en todo el espectro de concentraciones de selección de dicho antibiótico. Pertrechados de este conocimiento, se podría dar un paso adelante en el entendimiento del papel que juegan los ecosistemas contaminados con concentraciones subletales de antibióticos en el problema universal que constituye la resistencia a éstos.

Aportaciones específicas:

Trabajo experimental: Sanz-García, F., Sánchez, M. B. y Hernando-Amado, S. contribuyeron a la labor experimental. Concretamente, mi participación se centró en el análisis bioinformático de los genomas secuenciados de las poblaciones evolucionadas, la confirmación de la existencia de las mutaciones identificadas, la cuantificación de la sensibilidad a antibióticos distintos al de selección en las poblaciones mentadas, la medición del nivel de expresión de *mexXY-oprM*, los ensayos de crecimiento y de frecuencia de mutación, y la interpretación de los resultados.

Elaboración del manuscrito: todos los autores contribuyeron a la escritura y corrección del manuscrito, llevando a cabo yo la primera versión del mismo.

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Evolutionary landscapes of *Pseudomonas aeruginosa* towards ribosome-targeting antibiotic resistance depend on selection strength

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ABSTRACT

It is generally accepted that antibiotic-resistant mutants are selected in a range of concentrations ranging from the minimum inhibitory concentration (MIC) to the mutant preventive concentration. More recently, it has been found that antibiotic-resistant mutants can also be selected at concentrations below MIC, which expands the conditions where this selection may occur. Using experimental evolution approaches followed by whole-genome sequencing, the current study compares the evolutionary trajectories of *Pseudomonas aeruginosa* in the presence of tobramycin or tigecycline at lethal and sublethal concentrations. Mutants were selected at sublethal concentrations of tigecycline (1/10 and 1/50 MIC), whereas no mutants were selected in the case of tobramycin, indicating that the width of sub-MIC selective windows is antibiotic-specific. In addition, the patterns of evolution towards tigecycline resistance depend on selection strength. Sublethal concentrations of tigecycline select mutants with lower tigecycline MICs and higher MICs to other antibiotics belonging to different structural families than mutants selected under lethal concentrations. This indicates that the strength of the cross-resistance phenotype associated with tigecycline resistance is decoupled from selection strength. Accurate information on the sublethal selection window for each antibiotic of clinical value, including the phenotypes of cross-resistance of mutants selected at each antibiotic concentration, is needed to understand the role of ecosystems polluted with different antibiotic concentrations in the selection of antibiotic resistance. Integration of this information into clinical and environmental safety controls may help to tackle the problem of antibiotic resistance.

1. Introduction

The antibiotic crisis cannot be understood without considering the most basic aspects of bacterial evolution. Besides being the cause of antibiotic resistance (AR), evolution should be considered as a 'passkey' for tackling this problem. Although evolution is sensitive to many unexpected factors and may be, in principle, stochastic and hence unpredictable, it is also known that different 'bottlenecks' may constrain the evolutionary process, hence moving evolution from stochasticity towards some determinism [1] and allowing a certain degree of prediction. This has been reflected in an increasing number of experimental evolution studies, which found that the diversity of evolutionary trajectories followed by bacterial populations in the presence of a selective pressure is limited [2,3]. The main factors that constrain mutational evolution towards resistance are the mutation supply rate, the level of resistance and differential fitness of the resistant mutants, and the strength of the selective pressure [4]. Several studies addressing the effect of mutation frequency (MF) [5], including analysis of hypermutators [3] and fitness costs [6,7], in the evolution of AR have been published, and the role of low-level AR in the evolution towards resistance has also been examined [8]. Although some studies have revealed the importance of low antibiotic concentrations in the selection of AR [9–11], information on the role of the strength of selection pressure on AR evolution is limited. In this regard, more detailed information on sublethal concentrations allowing AR selection by different antibiotics, as well as on the possibility that selection strength may modulate the genotypic and phenotypic (including cross-resistance) evolutionary trajectories or type of resistant mutants, is still needed [9,11,12].

It is generally accepted that selection of resistant mutants by an antibiotic occurs in a narrow range of concentrations – the 'selection window' [13] – spanning from the minimum inhibitory

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concentration (MIC) for a wild-type strain to the mutant preventive concentration which inhibits the growth of single-step resistant mutants. More recent studies have shown that the selection of resistant mutants, even those with high levels of resistance, may also arise at sublethal antibiotic concentrations [9,10]. Since low-level resistant mutants are only selectable at low antibiotic concentrations, it could be expected that a greater diversity of mutants would be selected at sublethal antibiotic concentrations than at lethal concentrations [14]. While some studies support this possibility [10], other studies have shown that the diversity of resistant mutants is greater under strong selective pressure [11].

Sublethal concentrations of antibiotics are present in clinical settings (i.e. in certain tissues or as a consequence of incomplete treatment or limited drug accessibility) and, importantly, in several natural environments (i.e. rivers, lakes, sewage water, sludge and even drinking water) [15]. These low antibiotic concentrations present in polluted natural environments are likely contributing to globalize the problem of AR through the selection of resistant bacteria that can potentially spread towards human hosts [16]. This situation can be particularly relevant in the case of pathogens with an environmental origin, such as *Pseudomonas aeruginosa*. This opportunistic pathogen, which is the main driver of morbidity and mortality in patients with cystic fibrosis and causes acute nosocomial infections [17], is distributed extensively in nature [18]. Its intrinsic low-level susceptibility to several drugs is of concern; in addition, it can achieve clinically relevant resistance by the acquisition of chromosomal mutations or through horizontal gene transfer of AR genes [19]. Therefore, the concentration ranges of drugs of clinical value that can select for AR in *P. aeruginosa*, as well as the level of resistance and collateral sensitivity acquired by the selected mutants, is of interest. This would enable prediction of whether the risk associated with the use of a particular drug is restricted to environments that contain high antibiotic concentrations (i.e. clinical settings) or if it extends to habitats with low antibiotic concentrations, including clinical and natural environments.

Using experimental evolution and whole-genome sequencing (WGS) approaches, the present authors have previously analysed the evolutionary trajectories of *P. aeruginosa* towards resistance in the presence of lethal concentrations of two ribosome-targeting antibiotics, namely tobramycin and tigecycline [2]. Despite both targeting the ribosome, their mechanisms of action differ. Tobramycin binds irreversibly to one of two aminoglycoside binding sites on the 30S ribosomal subunit, preventing formation of the 70S complex, and may also destabilize the bacterial membrane by binding to 16S rRNA [20]. Tigecycline inhibits protein translation by binding to the 30S ribosomal subunit and blocking the entry of amino-acyl tRNA molecules into the A site of the ribosome, preventing incorporation of amino acid residues into elongating peptide chains [21]. Tobramycin is included in usual therapy regimens against this pathogen [22], whereas *P. aeruginosa* is resistant to tigecycline at the concentrations used in therapy; as such, tigecycline is not used to treat *P. aeruginosa* infections. However, *P. aeruginosa* frequently re-infects nosocomial patients treated with tigecycline; consequently, populations of this pathogen can be under tigecycline challenge [23]. Although, to the best of the authors' knowledge, information about the amount of tigecycline present in natural environments has not been established, the fact that all antibiotics used for therapy or farming are released regularly into water and soil suggests that this antibiotic will be present in habitats regularly colonized by *P. aeruginosa*, which has an environmental origin [18]. Moreover, tigecycline has been repurposed to be used, instead of benzodiazepines, for the treatment of alcohol disorders [24]; this may increase the circumstances where this antibiotic can exert selection compared with its use for treating infections [23]. This study investigates if the presence of two different sub-MIC concentrations of either tigecycline or tobramycin might select resistant mutants, and whether or not these mutants, if selected, present collateral sensitivity or cross-resistance to antimicrobial agents used in clinics.

This study provides comprehensive information on alternative evolutionary routes that *P. aeruginosa* may follow in environments where different concentrations of ribosome-targeting antibiotics of clinical value might be present.

2. Materials and methods

2.1. Experimental evolution and antibiotic susceptibility assays

This experimental evolution study was performed at the same time, and using the same batch of medium, as the authors' previous study using lethal concentrations [2]. Twenty bacterial populations from a stock of *P. aeruginosa* PA14 were grown in parallel in Mueller Hinton (MH) broth (Condalab, Torrejon de Ardoz, Spain) at 37°C with shaking at 250 rpm for 35 consecutive days. Four controls were grown without antibiotic, and the remaining populations were grown in the presence of sublethal concentrations of either tobramycin or tigecycline. For each antibiotic, four populations were challenged with a concentration of 1/10 MIC and four populations were challenged with a concentration of 1/50 MIC (tigecycline MIC = 4 μg/mL, tobramycin MIC = 0.5 μg/mL). Each day, the cultures were diluted (1/250) in fresh MH broth. The MICs of tigecycline and tobramycin were ascertained every 5 days using MIC test strips (Liofilchem, Waltham, MA, USA) at 37°C in MH agar plates, and each replicate population was preserved at -80 °C.

Susceptibility to a broad range of antibiotics (tetracycline, aztreonam, imipenem, ceftazidime, ciprofloxacin, levofloxacin, amikacin, tobramycin, chloramphenicol, erythromycin and fosfomycin) was determined in the final evolved populations in which an increased level of resistance to the antibiotic used for selection was observed during evolution, using the conditions indicated above.

2.2. Growth measurement

A 10 μL sample of *P. aeruginosa* PA14 overnight culture was inoculated in 140 μL of MH medium in the presence of 1/10 and 1/50 MIC of tigecycline and tobramycin and in the absence of antibiotic to a final optical density (OD_{600}) of 0.01. Growth (OD_{600}) of three independent replicates, in a 96-well microtitre plate (Nunclon Delta Surface; ThermoFisher Scientific, Waltham, MA, USA), was monitored every 10 min using a Spark 10M Plate Reader (Tecan, Männedorf, Switzerland) for 30 h at 37°C. Shaking was performed for 5 s before each measurement.

2.3. Determination of mutation frequency

The method used to assess MF has been reported elsewhere [25]. *P. aeruginosa* PA14 strain was grown overnight in Luria Bertani (LB) broth in the presence of 1/10 and 1/50 MIC of tigecycline and tobramycin and in the absence of antibiotic at 37°C with shaking at 250 rpm. One hundred microlitres of a 10^{-7} inoculum of each overnight culture (OD_{600} = 4) was seeded in LB agar, and 200 μL of a 10^{-2} inoculum was plated in LB agar containing 300 μg/mL rifampicin (Calbiochem, San Diego, CA, USA), a concentration that hinders the growth of *P. aeruginosa* PA14. After 24 h of incubation at 37°C, the number of colony-forming units was counted, and MF was established as the ratio between colonies on rifampicin plates and LB agar. Three replicates of each growth condition were included in the assay.

2.4. Whole-genome sequencing and bioinformatics analysis

Genomic DNA of each population that had experienced a decrease in susceptibility to tobramycin or tigecycline after 35 days

Table 1
Primers used to verify nucleotide modifications detected by whole-genome sequencing in populations evolved in sublethal concentrations of tigecycline, and to perform real-time reverse transcriptase polymerase chain reaction (RT-PCR).

Gene	Genetic change	Localization	Forward primer (5'–3')	Reverse primer (5'–3')
fleQ	A–>C	809	CTTCACCGGTGCCATCACCA	AGCAGGGCGATGTCTTCCAC
	T–>C	905		
	Del-A	184	GCCTGAGCAACAGTCGCGAA	GGCCAGTTGCTTCAGCAGCT
orfN	Ins-G	138	ATGGACGTTCCCAATGCCCG	CCGCCAGAATCAGCAAAACC
flgE	G–>C	1069	CAACGTGATCCAGTTCAGCC	CCTGGATGTTGGCGAAGTTC
fleS	C–>T	1112	CCTGGGCGAACCCTTCTTCA	AGGATCAAGGTGGCGCAGGT
pilQ2	A–>C	301	ACCAGCTTTACCGCGCCGTC	AAATGCACGTCACTGGCGCG
mraW	Del-A	13	CGGACGAACTACGCGACCTGAT	TACCGTCCAGATAGCAGCCGTC
Intergenic *mexZ-mexX*	Del-T	3421590	TCCATTGGATGTGCATG	AGTGGTGCCCACGCCCTT
bacA	G–>A	496	CTGAAGATCGGCTGTGCCCA	AGGTCGCGGTACTTGTAGCC
	G–>A	539		
	C–>T	50	TGGAGTGGTGGACTGCTTTC	GTCGGCGACGATGATCTGGT
	G–>A	49		
fleR	Del 14 bp	1015	TGTCGGTCTTCCCGCTG	CGCGGTGAGGCACAGGT
Real-time RT-PCR primers				
rplU	-	-	CGCAGTGATTGTTACCG GTG	AGGCCTGAATGCCGGTG ATC
mexX	-	-	GTACGAGGAAGGCCAGGAC	CTTGATCAGGTCGGCGTAG

Del, deletion; Ins, insertion.
The locations of all mutations refer to the specific gene in which they are located, except for the genetic modification in the intergenic region for which the location refers to the nucleotide position in the *Pseudomonas aeruginosa* UCBPP-PA14 reference chromosome (NC_008463.1).

Table 2
Minimum inhibitory concentrations (MICs) (μg/mL) for population replicates during experimental evolution under sub-MICs (1/10 and 1/50 MIC) and increasing inhibitory concentrations (from MIC to 32xMIC) of tigecycline and tobramycin.

Treatment	Replicate	5 d	10 d	15 d	20 d	25 d	30 d	35 d
Tigecycline 1/50 MIC	1	2	12	16	8	12	12	16
	2	2	12	16	24	16	24	16
	3	2	12	12	12	24	32	24
	4	2	8	12	8	12	24	24
Tigecycline 1/10 MIC	5	8	8	8	8	12	16	24
	6	12	12	12	16	32	32	32
	7	12	6	6	8	8	8	12
	8	8	8	8	12	12	12	16
Tigecycline MIC to 32xMIC	I5	32	≥256	≥256	≥256	≥256	≥256	≥256
	I6	48	≥256	≥256	≥256	≥256	≥256	≥256
	I7	48	32	≥256	≥256	≥256	≥256	≥256
	I8	32	≥256	≥256	≥256	≥256	≥256	≥256
Tobramycin 1/50 MIC	13	1	1	1	1	1	1	1
	14	0.75	1	1	1	1	1	1
	15	1	1	1	1	1	1	1.5
	16	1	1	1.5	1	1	1	1.5
Tobramycin 1/10 MIC	17	1	1.5	1.5	1	1.5	1	1
	18	1	1	1	0.75	1.5	1	1
	19	1	1	1.5	1	1.5	0.75	1
	20	1	1	1	0.75	1.5	1.5	1
Tobramycin MIC to 32xMIC	I1	6	6	12	16	24	24	24
	I2	4	4	8	8	24	24	24
	I3	4	4	6	12	16	32	24
	I4	4	6	12	16	24	32	32
MH medium without antibiotic	9	2–1	2–1	2–1.5	2–1	2–1	2–1	2–1.5
	10	2–1	2–1	2–1.5	2–1	2–1	2–1.5	2–1.5
	11	2–1	2–1.5	2–1.5	2–1.5	2–1	2–1.5	2–1.5
	12	2–1.5	2–1	2–1.5	2–1.5	2–1	2–1.5	2–1.5

MH, Mueller Hinton.
The table shows the MIC values of tigecycline and tobramycin for each replicate population every 5 days. Data for inhibitory evolution were extracted from the authors' previous study [2]. The populations that evolved under inhibitory conditions are named I1–4 for tobramycin and I5–8 for tigecycline. The MICs for tigecycline and tobramycin in the control populations grown in MH medium without antibiotic are indicated as two values separated by a dash (X–X), with the first value for tigecycline and the second value for tobramycin.

of evolution, as well as genomic DNA from the control populations, was extracted with the Gnome DNA kit (MP Biomedicals, Santa Ana, CA, USA), and WGS was performed by Sistemas Genomicos S.L (Paterna, Spain). The conditions followed for sequencing were the same as described elsewhere [2].

WGS data were analysed using CLC Genomics Workbench 12.0 (QIAGEN, Hilden, Germany), with *P. aeruginosa* UCBPP-PA14 as the reference chromosome (GenBank accession number NC_008463) used to align the reads, which had been trimmed previously. Mutations identified in the DNA samples obtained from populations grown under sublethal tigecycline concentrations were compared with those present in control populations.

2.5. Verification of single nucleotide polymorphisms

The presence of each putative mutation was verified by polymerase chain reaction (PCR) using the corresponding pairs of primers (Table 1). DNA fragments were purified using the QIAquick PCR Purification kit (QIAGEN) and Sanger sequencing was performed at GATC Biotech.

2.6. RNA preparation and real-time reverse transcriptase polymerase chain reaction

Twenty millilitres of MH broth were inoculated with an overnight culture of the selected clones from the final evolved populations and *P. aeruginosa* PA14 to a final OD_{600} of 0.01, and incubated until the exponential phase was reached (OD_{600} = 0.6). Ten millilitres of each sample were spun at 7000 rpm at 4 °C for 15 min. Three independent biological replicates were included in the experimental procedure.

Subsequently, the RNeasy Kit (QIAGEN) extraction protocol was performed to extract RNA from each sample. In order to remove any residual DNA, two DNase treatment protocols were followed with DNase I (QIAGEN) and TURBO DNase (Ambion, Austin, TX, USA). To check that no residual DNA was left in the samples, PCR with *rplU* primers (Table 1) was performed. A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize cDNA from 5 µg of RNA. Afterwards, real-time reverse transcriptase polymerase chain reaction was performed using 50 ng of cDNA and the Power SYBR Green Kit (Applied Biosystems) in an ABI PRISM 7500 real-time PCR system (Applied Biosystems). The expression level of *mexX* gene was elicited using the primers encompassed in Table 1, and all data were normalized using the gene *rplU*. The relative amounts of mRNA were measured following the $2^{-\Delta\Delta Ct}$ method described elsewhere [26].

3. Results

3.1. Lethal and sublethal concentrations of ribosome-targeting antibiotics drive evolution of P. aeruginosa towards distinct resistance levels

To determine if sublethal concentrations of tigecycline or tobramycin may select for resistance to these antibiotics in *P. aeruginosa*, an adaptive laboratory evolution (ALE) assay in the presence of two concentrations of these drugs (1/10 and 1/50 MIC of parental strain) was performed. As shown in Fig. 1 and Table 2, a notable increase in the MIC of tigecycline was observed for both sublethal treatments after 35 days of evolution, without noticeable disparities between the levels acquired by the populations evolving under either 1/10 or 1/50 sub-MIC conditions (from 6-fold to 16-fold of MIC of the parental strain). Taking into account that an increase in MIC of an antibiotic after experimental evolution may be due to a phenotypic adaptation to the presence of the antibiotic rather than to mutations [27], the evolved populations were subcultured without selection pressure (three sequential passages) and MICs were examined again; no variation was identified.

These results indicate that sublethal concentrations of tigecycline may select for resistant mutants, although their MIC values are lower than those reached in the presence of lethal concentrations of tigecycline (128-fold of wild-type MIC) (Table 2). It has been reported that sublethal concentrations of antibiotics may also increase the mutation rate, hence favouring the emergence of mutants [5]. To address this possibility, the MFs of *P. aeruginosa* PA14 populations that had grown overnight in medium without antibiotic or in the presence of either 1/10 or 1/50 MIC of tigecycline or tobramycin were compared. Fig. 2 shows that MF increased upon challenging with sublethal concentrations of tigecycline compared with no antibiotic (4.8-fold and 3.6-fold for 1/10 and 1/50 MIC, respectively); no differences were observed in the case of tobramycin.

In contrast to the situation observed with tigecycline, the *P. aeruginosa* populations submitted to ALE in the presence of 1/10 or 1/50 sub-MIC concentrations of tobramycin did not have an increased MIC (Fig. 1 and Table 2). Consequently, these populations were not analysed further. As selection at sublethal concentrations is based on the differential fitness of wild-type and mutant strains under selective pressure [12], it is possible that the effects of the two antibiotics on bacterial fitness are different. Indeed, the present results show that sublethal concentrations of tigecycline, particularly 1/10 MIC, seriously affected the fitness of the populations, decreasing the bacterial growth rate and final OD_{600} in the stationary phase. Conversely, sublethal concentrations of tobramycin did not alter bacterial growth (Fig. 3). These results indicate that the mutational space for tobramycin is reduced compared with that for tigecycline, most likely because the effects of sublethal concentrations of these antibiotics on bacterial growth differ. Consequently, the chance of selecting mutants in clinical and non-clinical habitats containing low concentrations of these antibiotics is lower for tobramycin than for tigecycline.

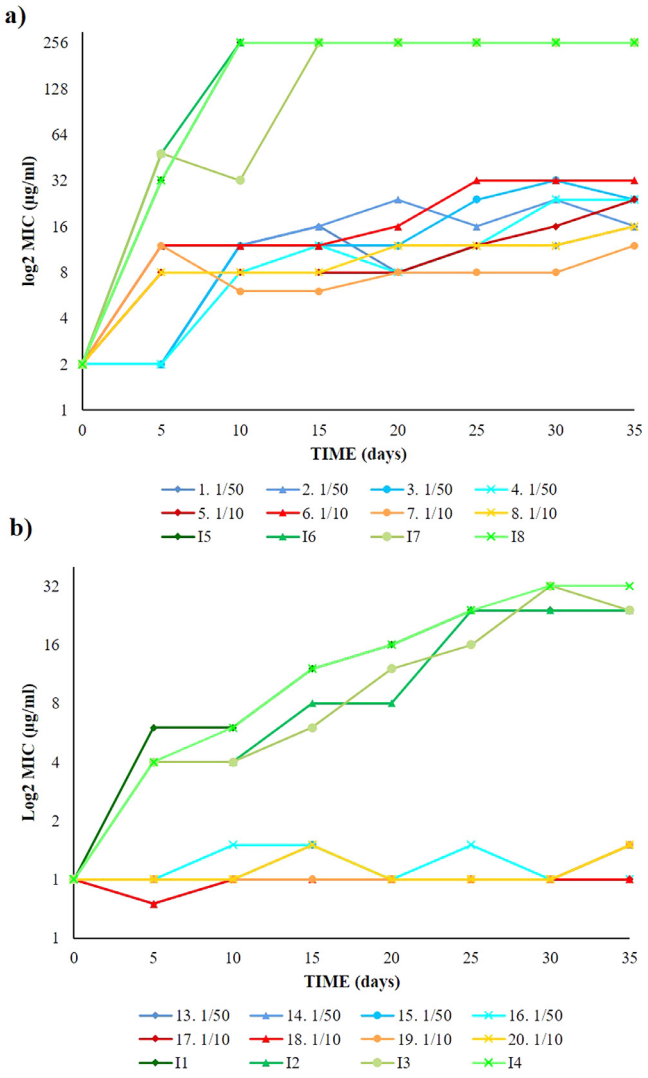

Fig. 1. Evolution of *Pseudomonas aeruginosa* under lethal and sublethal concentrations of ribosome-targeting antibiotics. Sub-minimum inhibitory concentrations (MICs) (1/50 and 1/10 MICs) and increasing inhibitory concentrations of (a) tigecycline and (b) tobramycin were used. Graphs show the changes in MICs (obtained by E-test strips) over the evolution period from the value corresponding to the wild-type strain (tigecycline = 2 µg/mL; tobramycin = 1 µg/mL) to different levels of resistance. MICs for each replicate and controls are provided in Table 2. Data for inhibitory evolution were extracted from the authors' previous study [2], and the populations are named I1–4 for tobramycin and I5–8 for tigecycline.

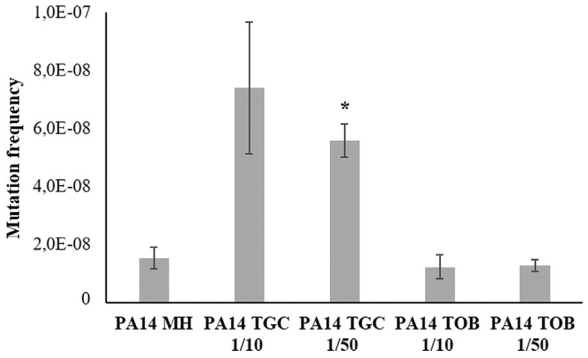

Fig. 2. Mutation frequency of *Pseudomonas aeruginosa* PA14 at sublethal concentrations of tigecycline (TGC) and tobramycin (TOB). The graph displays the mutation frequency of *P. aeruginosa* PA14 strain grown in broth containing 1/10 or 1/50 of the minimum inhibitory concentration (MIC) of TGC or TOB, or in the absence of antibiotic. Error bars indicate standard deviations of the results from three independent experiments. Significant differences ($P<0.05$) in the mutation frequency of *P. aeruginosa* PA14 grown in Mueller Hinton medium containing sublethal concentrations of TGC or TOB compared with *P. aeruginosa* PA14 grown in the absence of antibiotic were evaluated using Student's *t*-test and are highlighted with an asterisk (*).

3.2. Cross-resistance and collateral sensitivity associated with tigecycline resistance are dependent on selection strength during evolution of P. aeruginosa

It has been reported previously that *P. aeruginosa* resistant mutants selected on increasing lethal concentrations of tigecycline (from MIC to 32xMIC) have cross-resistance to other antibiotics besides tigecycline [2]. In order to compare cross-resistance and collateral sensitivity patterns associated with the evolution of tigecycline resistance under lethal and sublethal conditions, MICs of a set of antibiotics were determined for the final evolved populations on 1/10 and 1/50 sub-MIC concentrations of tigecycline and compared with those detected previously at lethal concentrations [2] (Fig. 4 and Table 3).

Every replicate from both ALE assays showed lower susceptibility to various antimicrobials (Fig. 4 and Table 3), especially quinolones, aminoglycosides and chloramphenicol. Important quantitative differences were found in the strength of phenotypic alterations between the replicates evolved under lethal or sublethal conditions. Populations that evolved under lethal conditions exhibited a stronger cross-resistance phenotype to tetracycline, aztreonam and ceftazidime compared with populations that evolved under sublethal conditions, which is in agreement with previous findings [11]. However, certain populations that evolved under sublethal concentrations of tigecycline had higher MICs for ciprofloxacin (Populations 6 and 8), imipenem (Populations 3 and 7), levofloxacin (all 1/10 populations and Population 4) and erythromycin (all populations) than populations that evolved under lethal concentrations of tigecycline (Fig. 4 and Table 3). The differences in levofloxacin susceptibility were particularly remarkable. Notably, the populations that evolved under 1/10 MIC of tigecycline exhibited a much higher increase in levofloxacin MIC – beyond the EUCAST clinical breakpoint (Table 3) – than the populations that evolved under either 1/50 MIC or lethal concentrations of tigecycline. It should be noted that while populations evolved under lethal concentrations of tigecycline were hypersusceptible to fosfomycin, this phenotype was not observed for populations that evolved under sublethal concentrations of tigecycline (Fig. 4 and Table 3). These results indicate that cross-resistance and collateral sensitivity of *P. aeruginosa* associated with tigecycline resistance is contingent on selection strength during evolution. Moreover, the data indicate that there is no quantitative correlation between the level of resistance to tigecycline and the level of cross-resistance to antibiotics belonging to different structural families.

Finally, it is worth highlighting that concentrations of tigecycline as low as 1/50 MIC may select for *P. aeruginosa* mutants with low susceptibility to clinically important antipseudomonal antimicrobials, such as amikacin, imipenem or quinolones [22].

3.3. Evolutionary landscapes of P. aeruginosa in the presence of sublethal concentrations of tigecycline

To gain insight into the genetic modifications associated with the acquisition of resistance in *P. aeruginosa* populations challenged with sublethal concentrations of tigecycline, their genomes, as well as those of the original PA14 strain and the control populations, were sequenced at the end of evolution (35 days). All control populations harboured a GA deletion in the gene that encodes the Quorum Sensing regulator LasR, and population 10 also presented a single nucleotide polymorphism (SNP) in *rpoB*; both genetic changes displayed coverage ranging from 73% to 85%. As these mutants were also selected in the absence of antibiotics, they were ruled out from further analysis as these mutations were considered to be involved in adaptation to the growth medium.

The presence of detected mutations (Table 4) was confirmed by PCR and Sanger sequencing using the oligonucleotides shown in Table 1. In total, 14 different genetic alterations (13 SNPs and one

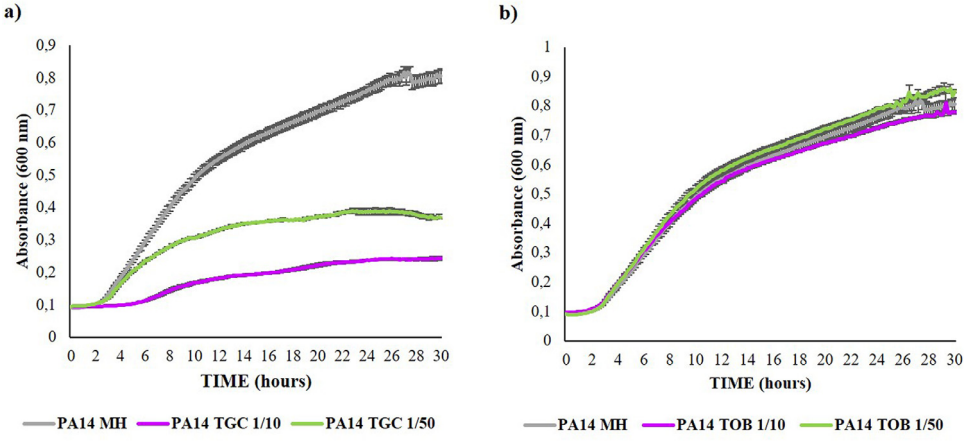

Fig. 3. *Pseudomonas aeruginosa* PA14 growth in the presence of sublethal concentrations of tigecycline (TGC) and tobramycin (TOB). The graphs show the growth of *P. aeruginosa* PA14 strain in Mueller Hinton (MH) medium and MH containing 1/10 and 1/50 MIC of (a) TGC and (b) TOB for 30 h at 37°C. Error bars indicate standard deviations of the results from three independent experiments.

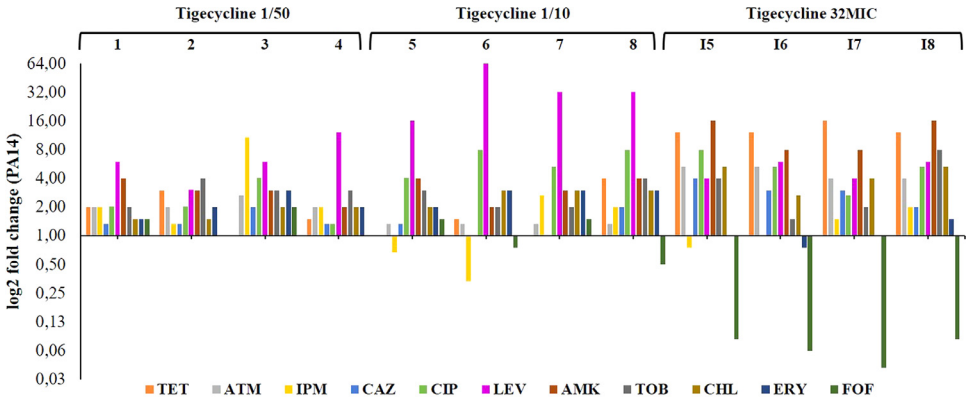

Fig. 4. Antimicrobial susceptibility of different families of *Pseudomonas aeruginosa* populations that evolved under lethal and sublethal concentrations of tigecycline. The graph shows the minimum inhibitory concentrations (MICs) of the populations that evolved under sub-MICs (1/10 and 1/50 MIC) and at lethal concentrations of tigecycline at the end of evolution (35 days). Data for populations that evolved under lethal concentrations were extracted from the authors' previous study [2]. MICs are displayed as $\log_2$ of the fold change of values for *P. aeruginosa* PA14 strain. MICs for each replicate and controls are provided in Table 3. TET, tetracycline; ATM, aztreonam; IPM, imipenem; CAZ, ceftazidime; CIP, ciprofloxacin; LEV, levofloxacin; AMK, amikacin; TOB, tobramycin; CHL, chloramphenicol; ERY, erythromycin; FOF, fosfomycin.

Table 3
Minimum inhibitory concentrations (MICs) (μg/mL) of antibiotics of different structural families in the final populations that evolved under sub-MIC (1/10 and 1/50 MIC) or inhibitory concentrations of tigecycline.

Replicate	TET	ATM	IPM	CAZ	CIP	LEV	AMK	TOB	CHL	ERY	FOF
PA14	16	1.5	0.75	0.75	0.094	0.125	2	0.5	32	32	32
EUCAST											
>R breakpoints	-	16	4	8	0.5	1	16	2	-	-	-
Tigecycline 1/50 MIC											
1	32	3	1.5	1	0.19	0.75	8	1	48	48	48
2	48	3	1	1	0.19	0.38	6	2	48	64	32
3	16	4	8	1.5	0.38	0.75	6	1.5	64	96	64
4	24	3	1.5	1	0.125	1.5	4	1.5	64	64	32
Tigecycline 1/10 MIC											
5	16	2	0.5	1	0.38	2	8	1.5	64	64	48
6	24	2	0.25	0.75	0.75	8	4	1	96	96	24
7	16	2	2	0.75	0.5	4	6	1	96	96	48
8	64	2	1.5	1.5	0.75	4	8	2	96	96	16
Tigecycline 32xMIC											
I5	192	8	0.5	4	0.75	0.5	32	4	128	32	2
I6	192	8	0.75	3	0.5	0.75	16	1.5	64	24	1.5
I7	256	6	1	3	0.25	0.5	16	2	96	32	1
I8	192	6	1.5	2	0.5	0.75	32	8	128	48	2
Controls											
9	12	2	0.75	1	0.094	0.19	2	0.75	24	32	32
10	16	2	0.75	1	0.19	0.125	2	0.75	16	24	32
11	12	2	0.75	1	0.094	0.125	3	0.75	32	32	32
12	12	2	0.75	0.75	0.125	0.125	3	0.75	16	48	32

TET, tetracycline; ATM, aztreonam; IPM, imipenem; CAZ, ceftazidime; CIP, ciprofloxacin; LEV, levofloxacin; AMK, amikacin; TOB, tobramycin; CHL, chloramphenicol; ERY, erythromycin; FOF, fosfomycin.
Data for inhibitory evolution were extracted from the authors' previous study [2], and the populations evolved under tigecycline inhibitory conditions are named I5–8. MIC breakpoints for the antibiotics included in EUCAST for *Pseudomonas aeruginosa* are encompassed in the table.

multi-nucleotide polymorphism) were sought, and the majority led to amino acid alterations or frameshifts.

First, mutations in flagellar genes such as *fleQ*, the genes that encode the two-component system FleRS, and FlgE, were selected and hence may have a role in the acquisition of resistance by populations 2, 5, and 7; 4 and 8; and 3, respectively. FleQ is the master regulator of flagellar biosynthesis, and is also involved in biofilm formation [28]. FleRS participates in regulation of motility and adhesion to mucin [29]. Concerning the latter, it has been demonstrated that inactivating mutations in *fleS* (different from that selected in the present experimental setting) led to hypersusceptibility to ciprofloxacin [30], while a mutation in *fleQ* was also selected in the authors' previous ALE study using lethal concentrations of tobramycin [2]. On the contrary, no evidence of involvement of the flagellar hook protein FlgE in drug resistance has been reported.

Mutations in *bacA*, a gene encoding a UDP pyrophosphate phosphatase, were selected for in three of the populations evolved under 1/10 MIC of tigecycline and in one population evolved under 1/50 MIC of tigecycline (Table 4). However, no link between *bacA* and AR has been reported to date. In contrast, mutations in *pilQ2* (which encodes a type IV B pilus protein) have been reported to be related to gonococcal AR because of a decrease in stability of the PilQ doughnut-like multimeric structure in the outer membrane which may reduce the entry of hydrophilic antibiotics, such as tigecycline [17]. Moreover, genetic changes in *pilQ* have also been identified in *P. aeruginosa* isolates from patients with cystic fibrosis, and in *P. aeruginosa* ALE studies in the presence of tobramycin [31] and at sublethal concentrations of ciprofloxacin [32]. This last study, together with the present results, suggests that *pilQ* may have a role in the response to common stresses, such as the production of reactive oxidants triggered in the presence of antibiotics [33]. In addition, a deletion in the intergenic region between the *mexXY* operon, which codes for a multidrug efflux pump, and the *mexZ* gene, which encodes its regulator, was also selected. MexXY

Table 4
Genetic modifications detected in *Pseudomonas aeruginosa* PA14 populations evolved in the presence of 1/10 or 1/50 of the minimum inhibitory concentration (MIC) of tigecycline.

Gene	Replicate	Mutation	Nucleotide localization[a]	Aa change	Coverage[b] (%)
Both sub-MIC concentrations					
pilQ2	2, 8	A–>C	301	Val50fs	91,39/74,24
fleQ	2	A–>C	809	Val270Gly	93,22
	5	Del-A	184	Ser62fs	61,78
	7	A–>G	905	Asp302Gly	99,32
bacA	1	G–>A	49	Gly17Arg	31,11
	5	T–>G	721	Thr241Pro	89,40
	6	G–>A	50	Gly17Glu	90
	7	C–>T	539	Ser180Leu	98,67
1/50 MIC					
flgE	3	G–>C	1069	Gly357Arg	34,34
fleS	4	G–>A	1112	Gly371Glu	69,47
1/10 MIC					
mraW	6	Del-T	19	Tyr7fs	61,74
orfN	8	Ins-G	138	Val50fs	55,03
fleR	8	Del-14 bp	1015	Val339fs	37,88
Intergenic mutation					
Intergenic *mexX-mexZ*	5	Del-T	3421590	-	84,78

fs, frameshift; Del, deletion; Ins, insertion.
[a] Nucleotide locations of mutations refer to the specific gene in which they are located and their associated amino acid changes. A genetic modification in an intergenic region is also included, and its location refers to the nucleotide position in *Pseudomonas aeruginosa* UCBPP-PA14 reference chromosome (NC_008463.1).
[b] Coverage stands for the percentage of reads of each mutant allele among the total number of reads corresponding to the same region in the genome, within the whole population, at the end of experimental evolution.

is known to participate in tigecycline extrusion, among other substrates [34]. To address the effect of this mutation in the resistance phenotype, two clones from the final population that contained the intergenic mutation (population 5) – one presenting the SNP and one with the wild-type genotype – were selected. No differences were ascertained in *mexX* expression between the two clones, suggesting that the selected mutation does not affect expression of this efflux pump. The mutation in *orfN*, which codes for a putative glycosyl transferase, appears to be important for *P. aeruginosa* motility [35] and AR due to its selection in previous studies of evolution in the presence of tigecycline, tobramycin, ceftazidime or ciprofloxacin [2,35–37]. Finally, *mraW* encodes an endogenous ribosomal methyltransferase [38], the mutation of which in one of the 1/10 MIC tigecycline populations (Table 4) could have an effect related to modification of the antibiotic's target.

It is important to note that most mutant alleles selected in the presence of antibiotic have coverage >50% and typically >80% (Table 4), indicating that they are under positive selection when *P. aeruginosa* grows in the presence of tigecycline, although it is possible that some mutations might be selected to compensate the fitness costs associated with the acquisition of AR. The percentages of mutant alleles in Populations 1 (mutation in *bacA*: 31.11%) and 3 (mutation in *flgE*: 34.34%) are <50%, suggesting that these populations are not homogeneous. In agreement with this feature, a double halo with scattered colonies inside was observed in their MIC test strips assays, corroborating that they are mixed populations with different MIC values.

3.4. Comparison of genetic modifications selected in P. aeruginosa under lethal and sublethal concentrations of tigecycline

As detailed above, the phenotypic evolution of *P. aeruginosa* towards AR when submitted to either lethal or sublethal concentrations of tigecycline differed markedly. This may be explained by different genotypic trajectories followed by the populations evolved under different conditions. Notably, and in line with previous work [10], populations challenged with lethal antibiotic concentrations acquired more mutations (Fig. 5). The higher variability of mutations selected under lethal concentrations may be due to different experimental settings; in the case of sub-MIC assays, long-term fixed antibiotic concentrations are expected to select for the fittest mutants (not just for those that allow survival), while stepwise increases in concentrations are expected to favour sequential accumulation of AR mutations. Further, the majority of mutations that arose under lethal conditions, such as those in *nfxB, mexC, mexD, parRS, rpsJ, orfN* or *pmrB*, were already known to be implicated in AR [2,35]. On the contrary, half of the genes presenting mutations selected at sublethal conditions have not been characterized previously as resistance determinants (*fleQ, fleR, flgE, bacA* or *mraW*). Whether or not this type of mutants constitutes a hidden part of the low-level *P. aeruginosa* resistome, and its clinical relevance, remain to be explored.

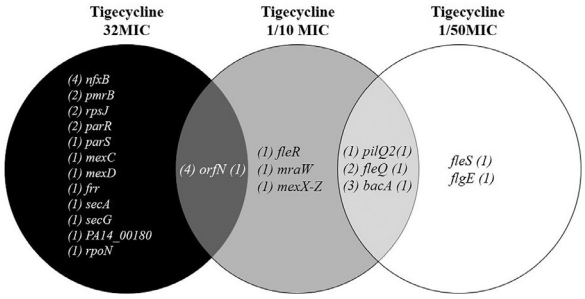

Fig. 5. Common and different genetic changes identified in *Pseudomonas aeruginosa* populations that evolved under lethal and sublethal concentrations of tigecycline. The figure shows the genes in which mutations were selected after 35 days of evolution in each of the sublethal experiments compared with those selected under inhibitory conditions [2]. The number of replicates containing mutations in each gene is shown in parentheses. MIC, minimum inhibitory concentration.

Only one gene, *orfN* (OrfNVal50fs), carried mutations that were selected for at high and low concentrations of tigecycline, and only one population harboured this mutation under the sub-MIC conditions studied (Fig. 5 and Table 4). The present results support the

importance of this mutation in the acquisition of tigecycline resistance by *P. aeruginosa* PA14.

4. Discussion

The finding that mild selection pressures (sublethal antibiotic concentrations) may select antibiotic-resistant mutants increases the range of habitats where AR can emerge. Besides the treated, infected host, these habitats encompass non-clinical ecosystems that can be polluted with antibiotics as wastewater. Fighting AR from a One-Health perspective [16] requires information on the sublethal concentrations that allow selection of resistant mutants, and on the cross-resistance phenotype of the selected mutants as a function of selection strength. This study found that the range of sublethal concentrations capable of selecting *P. aeruginosa* antibiotic-resistant mutants differs for two ribosome-targeting antibiotics – tobramycin and tigecycline – with a larger range for tigecycline. The fact that resistant mutants are not selected at the tested concentrations of tobramycin is relevant as other antimicrobial agents, such as ciprofloxacin, have been shown to select for resistant mutants at concentrations as low as 1/230 of wild-type MIC [9]. Indeed, although it has been reported that *P. aeruginosa* antibiotic-resistant mutants can be selected using 1/2 MIC of tobramycin [39], no mutants were selected in the present study using 1/10 MIC of tobramycin. It has been stated that fitness in the presence of the selecting antibiotic is the major force for selecting antibiotic-resistant mutants at sublethal concentrations of antibiotics [12]. The fact that the effect of these antibiotic concentrations on the growth of *P. aeruginosa* was high for tigecycline and nearly absent for tobramycin (Fig. 3) shows that resistant mutants are easily selected at low concentrations of tigecycline but not of tobramycin. Further, MF in the presence of tigecycline increased to levels corresponding to a weak mutator [40], but did not change in the presence of tobramycin. This may also favour the selection of antibiotic-resistant mutants at sublethal concentrations of tigecycline.

It is worth mentioning that mutants selected under sublethal concentrations, which can be present in different habitats as well as in clinical settings, present cross-resistance to other antibiotics. Hence, at least in terms of their role as AR selectors, 'safe antibiotic concentrations' in natural ecosystems (including wastewater treatment plants, where antibiotics used for therapy are released) must be defined, taking into account that the selection window is antibiotic-specific. In the case of tigecycline and tobramycin, it is possible to predict the levels of resistance, cross-resistance and collateral sensitivity which might be selected under specific concentrations of these ribosome inhibitors. Notably, although the evolutionary trajectories towards tigecycline resistance in *P. aeruginosa* may differ at both phenotypic and genotypic levels depending on selection strength, both lethal and sublethal concentrations of tigecycline (even 1/50 MIC of *P. aeruginosa*) may select resistance to other clinically relevant antibiotics in this opportunistic pathogen. It may be thought that this bacterial species will rarely be confronted with this selective pressure as tigecycline is not used for treating *P. aeruginosa* infections, and is mainly used in re-infected patients [23]. However, sublethal concentrations of tigecycline released from treated patients may be found in environments colonized by *P. aeruginosa* as well as clinical settings. In these circumstances, selection of resistant *P. aeruginosa* mutants against several antimicrobials of clinical importance, such as quinolones, may occur in non-clinical ecosystems. As tigecycline is not used to treat *P. aeruginosa*, this is a proof-of-concept showing that the collateral effects of evolution in the presence of a given antibiotic (as cross-resistance to clinically relevant antibiotics) may be even more relevant than the primary effects (in this case, tigecycline resistance).

These results indicate that mild selective pressure by antibiotics encountered by bacteria, in clinical settings and also as pollutants in non-clinical ecosystems, can be a risk for the selection of multidrug-resistant bacteria.

Declarations

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References

[1] Rouzine IM, Rodrigo A, Coffin JM. Transition between stochastic evolution and deterministic evolution in the presence of selection: general theory and application to virology. Microbiol Mol Biol Rev 2001;65:151–85.
[2] Sanz-Garcia F, Hernando-Amado S, Martinez JL. Mutational evolution of *Pseudomonas aeruginosa* resistance to ribosome-targeting antibiotics. Front Genet 2018;9:451.
[3] Ibacache-Quiroga C, Oliveros JC, Couce A, Blázquez J. Parallel evolution of high--level aminoglycoside resistance in *Escherichia coli* under low and high mutation supply rates. Front Microbiol 2018(9):427.
[4] Hughes D, Andersson DI. Evolutionary trajectories to antibiotic resistance. Ann Rev Microbiol 2017;71:579–96.
[5] Blazquez J, Rodriguez-Beltran J, Matic I. Antibiotic-induced genetic variation: how it arises and how it can be prevented. Ann Rev Microbiol 2018;72:209–30.
[6] Praski Alzrigat L, Huseby DL, Brandis G, Hughes D. Fitness cost constrains the spectrum of marR mutations in ciprofloxacin-resistant *Escherichia coli*. J Antimicrob Chemother 2017;72:3016–25.
[7] Olivares Pacheco J, Alvarez-Ortega C, Alcalde Rico M, Martinez JL. Metabolic compensation of fitness costs is a general outcome for antibiotic-resistant *Pseudomonas aeruginosa* mutants overexpressing efflux pumps. mBio 2017(8):e0050017.
[8] Baquero F. Low-level antibacterial resistance: a gateway to clinical resistance. Drug Resist Update 2001;4:93–105.
[9] Gullberg E, Cao S, Berg OG, Ilback C, Sandegren L, Hughes D, et al. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog 2011;7:e1002158.
[10] Wistrand-Yuen E, Knopp M, Hjort K, Koskiniemi S, Berg OG, Andersson DI. Evolution of high-level resistance during low-level antibiotic exposure. Nat Commun 2018;9:1599.
[11] Oz T, Guvenek A, Yildiz S, Karaboga E, Tamer YT, Mumcuyan N, et al. Strength of selection pressure is an important parameter contributing to the complexity of antibiotic resistance evolution. Mol Biol Evolut 2014;31:2387–401.
[12] Hughes D, Andersson DI. Selection of resistance at lethal and non-lethal antibiotic concentrations. Curr Opin Microbiol 2012;15:555–60.
[13] Zhao X, Drlica K. Restricting the selection of antibiotic-resistant mutant bacteria: measurement and potential use of the mutant selection window. J Infect Dis 2002;185:561–5.
[14] Martinez JL, Baquero F. Mutation frequencies and antibiotic resistance. Antimicrob Agents Chemother 2000;44:1771–7.
[15] Danner MC, Robertson A, Behrends V, Reiss J. Antibiotic pollution in surface fresh waters: occurrence and effects. Sci Total Environ 2019;664:793–804.
[16] Hernando-Amado S, Coque TM, Baquero F, Martinez JL. Defining and combating antibiotic resistance from One Health and Global Health perspectives. Nat Microbiol 2019;4:1432–42.
[17] Zhao S, Tobiason DM, Hu M, Seifert HS, Nicholas RA. The penC mutation conferring antibiotic resistance in *Neisseria gonorrhoeae* arises from a mutation in the PilQ secretin that interferes with multimer stability. Mol Microbiol 2005;57:1238–51.
[18] Morales G, Wiehlmann L, Gudowius P, van Delden C, Tummler B, Martinez JL, et al. Structure of *Pseudomonas aeruginosa* populations analyzed by single nucleotide polymorphism and pulsed-field gel electrophoresis genotyping. J Bacteriol 2004;186:4228–37.
[19] Breidenstein EB, de la Fuente-Nunez C, Hancock RE. *Pseudomonas aeruginosa*: all roads lead to resistance. Trends Microbiol 2011;19:419–26.

[20] Le Goffic F, Capmau ML, Tangy F, Baillarge M. Mechanism of action of aminoglycoside antibiotics. Binding studies of tobramycin and its 6′-N-acetyl derivative to the bacterial ribosome and its subunits. Eur J Biochem 1979;102:73–81.
[21] Greer ND. Tigecycline (Tygacil): the first in the glycylcycline class of antibiotics. Proc (Bayl Univ Med Cent) 2006;19:155–61.
[22] Bassetti M, Vena A, Croxatto A, Righi E, Guery B. How to manage *Pseudomonas aeruginosa* infections. Drugs Context 2018;7:212527.
[23] Ulu-Kilic A, Alp E, Altun D, Cevahir F, Kalin G, Demiraslan H. Increasing frequency of *Pseudomonas aeruginosa* infections during tigecycline use. J Infect Dev Ctries 2015;9:309–12.
[24] Oliveros A, Choi DS. Repurposing tigecycline for the treatment of alcohol use disorder. Alcohol Clin Exp Res 2017;41:497–500.
[25] Oliver A, Sanchez JM, Blazquez J. Characterization of the GO system of *Pseudomonas aeruginosa*. FEMS Microbiol Lett 2002;217:31–5.
[26] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402–8.
[27] Levin BR, Rozen DE. Non-inherited antibiotic resistance. Nat Rev Microbiol 2006;4:556–62.
[28] Matsuyama BY, Krasteva PV, Baraquet C, Harwood CS, Sondermann H, Navarro MV. Mechanistic insights into c-di-GMP-dependent control of the biofilm regulator FleQ from *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 2016;113:E209–18.
[29] Dasgupta N, Wolfgang MC, Goodman AL, Arora SK, Jyot J, Lory S, et al. A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. Mol Microbiol 2003;50:809–24.
[30] Breidenstein EB, Khaira BK, Wiegand I, Overhage J, Hancock RE. Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. Antimicrob Agents Chemother 2008;52:4486–91.
[31] Lopez-Causape C, Rubio R, Cabot G, Oliver A. Evolution of the *Pseudomonas aeruginosa* aminoglycoside mutational resistome in vitro and in the cystic fibrosis setting. Antimicrob Agents Chemother 2018;62:e0258317.
[32] Ahmed MN, Porse A, Sommer MOA, Hoiby N, Ciofu O. Evolution of antibiotic resistance in biofilm and planktonic *Pseudomonas aeruginosa* populations exposed to subinhibitory levels of ciprofloxacin. Antimicrob Agents Chemother 2018;62:e0032018.
[33] Dwyer DJ, Collins JJ, Walker GC. Unraveling the physiological complexities of antibiotic lethality. Annu Rev Pharmacol Toxicol 2015;55:313–32.
[34] Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2000;44:3322–7.
[35] Hernando-Amado S, Sanz-Garcia F, Martinez JL. Antibiotic resistance evolution is contingent on the quorum sensing response in *Pseudomonas aeruginosa*. Mol Biol Evol 2019;36:2238–51.
[36] Sanz-Garcia F, Hernando-Amado S, Martinez JL. Mutation-driven evolution of *Pseudomonas aeruginosa* in the presence of either ceftazidime or ceftazidime-avibactam. Antimicrob Agents Chemother 2018;62:e0137918.
[37] Wong A, Rodrigue N, Kassen R. Genomics of adaptation during experimental evolution of the opportunistic pathogen *Pseudomonas aeruginosa*. PLoS Genet 2012;8:e1002928.
[38] Kimura S, Suzuki T. Fine-tuning of the ribosomal decoding center by conserved methyl-modifications in the *Escherichia coli* 16S rRNA. Nucl Acids Res 2010;38:1341–52.
[39] Wu YL, Scott EM, Po AL, Tariq VN. Development of resistance and cross-resistance in *Pseudomonas aeruginosa* exposed to subinhibitory antibiotic concentrations. Acta Pathol Microbiol Immunol Scand 1999;107:585–92.
[40] Mandsberg LF, Ciofu O, Kirkby N, Christiansen LE, Poulsen HE, Hoiby N. Antibiotic resistance in *Pseudomonas aeruginosa* strains with increased mutation frequency due to inactivation of the DNA oxidative repair system. Antimicrob Agents Chemother 2009;53:2483–91.

Artículo V

Mutation-driven evolution of *Pseudomonas aeruginosa* in the presence of either ceftazidime or ceftazidime-avibactam

Sanz-García, F., Hernando-Amado, S., & Martínez, J. L.

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Entre los tratamientos más candentes que a día de hoy se perfilan para combatir al patógeno oportunista *P. aeruginosa*, algunas combinaciones de β-lactámico-inhibidor de β-lactamasas brillan con luz propia, siendo éste el caso de la ceftazidima-avibactam. Esta terapia dual se encuentra hogaño circunscrita a casos clínicos muy delicados, entre los que se cuentan los pacientes con FQ infectados por cepas de *P. aeruginosa* multirresistentes, situación en la que dicha resistencia suele fraguarse vía mutaciones. Ergo, en este trabajo se recurrió de nuevo a la ALE a fin de desvelar los mecanismos de resistencia de esta bacteria frente a la ceftazidima y su combinación con avibactam.

Este experimento se llevó a cabo con *P. aeruginosa* PA14, sometida a concentraciones crecientes de cada uno de los dos tipos de presión selectiva durante 30 días, para finalmente desvelar, mediante secuenciación de genomas completos, las mutaciones de las poblaciones evolucionadas. Asimismo, fenotípicamente se constató que todas las poblaciones alcanzaron un nivel de resistencia a ceftazidima similar, a la par que resistencia cruzada a otros β-lactámicos, sensibilidad colateral a amikacina e hiper-producción del pigmento piomelanina. Estos tres rasgos se correlacionaron con la presencia de grandes deleciones (desde 55 kb hasta 442 kb) en el cromosoma de todas las réplicas. Con dichas deleciones se perdían los genes *galU* -lo que provoca resistencia a β-lactámicos-, *hmgA* -cuya eliminación dispara la acumulación de piomelanina-, y la bomba de expulsión MexXY -un importante determinante de resistencia a aminoglicósidos-. Además, el análisis de las restantes mutaciones situó el foco sobre la sobre-expresión del operón codificante del sistema de bombeo MexAB-OprM, preclaro responsable de la resistencia a β-lactámicos; los genes *mpl* y *dacB*, cuya mutaciones estimularon la actividad β-lactamasa (sólo seleccionadas en la ALE con ceftazidima); y mutaciones en genes codificantes de una bomba de expulsión RND y un sistema de dos componentes (ambos putativos), cuya proximidad podría sugerir la regulación de la primera por parte del segundo.

En síntesis, estos resultados enfatizan algunos de los mecanismos de resistencia por mutación que *P. aeruginosa* puede adquirir para arrostrar la terapia con ceftazidima y ceftazidima-avibactam. Asimismo, a sabiendas de que las cepas piomelanogénicas poseedoras de grandes deleciones son relativamente frecuentes en pacientes con FQ, debería tenerse en consideración que otros aspectos de su fenotipo podrían asemejarse a lo aquí descrito. Si así fuera, la amplia resistencia a β-lactámicos e hipersensibilidad a aminoglicósidos observadas en estos mutantes serían elementos críticos a valorar de cara a perfilar la terapia idónea contra estas infecciones.

Aportaciones específicas:

Trabajo experimental: Sanz-García, F. y Hernando-Amado, S. contribuyeron a la labor experimental. Concretamente, mi participación se centró en la elaboración de las ALEs, el análisis bioinformático de los genomas secuenciados de las poblaciones evolucionadas, la confirmación de la existencia de las mutaciones identificadas y determinación de su orden de aparición, la cuantificación de la sensibilidad a antibióticos distintos al de selección en las poblaciones mentadas, la medición de la actividad β-lactamasa y la interpretación de los resultados.

Elaboración del manuscrito: todos los autores contribuyeron a la escritura y corrección del manuscrito, llevando a cabo yo la primera versión del mismo.

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Mutation-Driven Evolution of *Pseudomonas aeruginosa* in the Presence of either Ceftazidime or Ceftazidime-Avibactam

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ABSTRACT Ceftazidime-avibactam is a combination of β-lactam/β-lactamase inhibitor, the use of which is restricted to some clinical cases, including cystic fibrosis patients infected with multidrug-resistant *Pseudomonas aeruginosa*, in which mutation is the main driver of resistance. This study aims to predict the mechanisms of mutation-driven resistance that are selected for when *P. aeruginosa* is challenged with either ceftazidime or ceftazidime-avibactam. For this purpose, *P. aeruginosa* PA14 was submitted to experimental evolution in the absence of antibiotics and in the presence of increasing concentrations of ceftazidime or ceftazidime-avibactam for 30 consecutive days. Final populations were analyzed by whole-genome sequencing. All evolved populations reached similar levels of ceftazidime resistance. In addition, they were more susceptible to amikacin and produced pyomelanin. A first event in this evolution was the selection of large chromosomal deletions containing *hmgA* (involved in pyomelanin production), *galU* (involved in β-lactams resistance), and *mexXY-oprM* (involved in aminoglycoside resistance). Besides mutations in *mpl* and *dacB* that regulate β-lactamase expression, mutations related to MexAB-OprM overexpression were prevalent. Ceftazidime-avibactam challenge selected mutants in the putative efflux pump *PA14_45890* and *PA14_45910* and in a two-component system (*PA14_45870* and *PA14_45880*), likely regulating its expression. All populations produced pyomelanin and were more susceptible to aminoglycosides, likely due to the selection of large chromosomal deletions. Since pyomelanin-producing mutants presenting similar deletions are regularly isolated from infections, the potential aminoglycoside hypersusceptiblity and reduced β-lactam susceptibility of pyomelanin-producing *P. aeruginosa* should be taken into consideration for treating infections caused by these isolates.

KEYWORDS *Pseudomonas aeruginosa*, avibactam, ceftazidime, mutational studies

Pseudomonas aeruginosa is an opportunistic pathogen widely distributed in nature (1) that is a major cause of lung and airway infections in hospitalized patients, as well of chronic infections in patients with cystic fibrosis (CF) and chronic obstructive pulmonary disease (2, 3). This bacterial species presents a characteristic low susceptibility to antibiotics, including β-lactams, which is mainly a consequence of its low permeability and the presence in its genome of different intrinsic resistance genes, including those encoding multidrug (MDR) efflux pumps (4) and β-lactamases. In addition, an increasing number of *P. aeruginosa* isolates have acquired several resistance genes through horizontal gene transfer (HGT), including those for resistance to different classes of carbapenemases. Finally, *P. aeruginosa* is able to develop resistance to nearly any available antibiotic through mutation, particularly when causing chronic infections. In this situation, the emergence and spread of MDR global clones are of special concern (5).

The use of β-lactam/β-lactamase inhibitor combinations, such as amoxicillin-clavulanic acid or ceftolozane-tazobactam, has been proven to be effective against class

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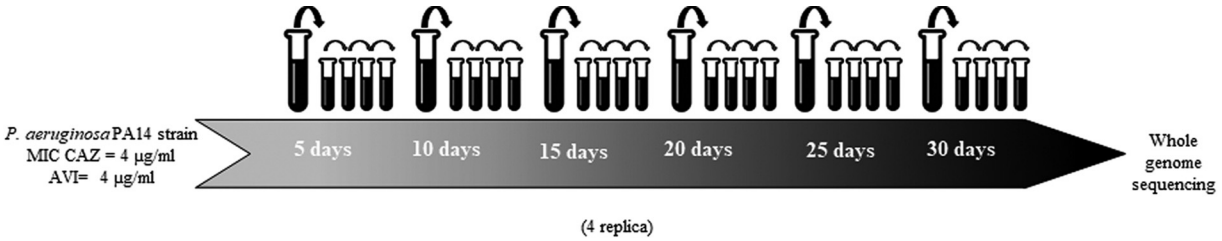

FIG 1 Experimental evolution assay. Eight bacterial cultures of *P. aeruginosa* strain PA14 were challenged with increasing inhibitory concentrations of ceftazidime in Luria Bertani broth (LBB) for 30 consecutive days. The ceftazidime concentration was raised by 2-fold every 5 days, from 4 μg/ml up to 32× MIC. Avibactam was added in combination with ceftazidime in four of these eight populations, at a constant concentration of 4 μg/ml, as this is the value established in clinical tests. Four controls without any selective pressure were also grown in parallel. At the end of the experimental evolution, the genomic DNA of the 12 independent populations was extracted and analyzed by whole-genome sequencing (WGS).

A β-lactamases (which include narrow- and extended-spectrum β-lactamases and some carbapenemases); whereas effective combinations against classes B, C (extended-spectrum cephalosporinases), and D β-lactamases (6–8) have not been available until recently. One of these is the ceftazidime-avibactam combination, the use of which was approved in 2015 by the FDA (9).

Avibactam, formerly known as NXL104, belongs to a new class of β-lactamase inhibitors, the diazabicyclooctanes (10). This inhibitor has a potent activity against most Class A and Class C and some Class D β-lactamases (11). Avibactam has been mainly used for restoring the activity of the third-generation cephalosporin ceftazidime (12). Thus far, it has been used for the treatment of patients with complicated urinary tract infections, including pyelonephritis, and for community-acquired intraabdominal infections, usually in combination with metronidazole (13). In addition, future studies are likely to expand the use of ceftazidime-avibactam to include other cases, such as those of cystic fibrosis patients with MDR *P. aeruginosa* infections (13).

Given the fact that this treatment is currently reserved for patients who have no alternative therapeutic options, a judicious use of antibiotic stewardship should be applied in order to prevent the incidence of drug resistance. Nevertheless, and although there are numerous studies on the activity of ceftazidime-avibactam against pathogens resistant to other antibiotics (14–17), analysis for predicting potential mechanisms of resistance to this antimicrobial combination are still scarce.

In the present study, experimental evolution followed by whole-genome sequencing (WGS) was used to examine the evolutionary trajectories taken by *P. aeruginosa* toward resistance against the combination ceftazidime-avibactam compared to the trajectories followed in the absence of avibactam. This may throw light upon the different mechanisms of resistance that are selected for in *P. aeruginosa* when its β-lactamase activity is inhibited by the presence of this novel inhibitor. In addition, the present work may allow us to elucidate whether the presence of avibactam modifies the resistance level acquired by the bacterial populations in comparison to the one developed when ceftazidime is used alone. Thus, these results may give rise to strategies for predicting, managing, and eventually reducing resistance to ceftazidime-avibactam. This is widely important, as this treatment is strictly restricted to few clinical cases in which resistant strains would be of major concern.

RESULTS

Stepwise evolution of *P.* aeruginosa toward ceftazidime and ceftazidime-avibactam resistance. To determine the potential evolutionary trajectories that can lead to either ceftazidime or ceftazidime-avibactam resistance, four biological replicates were allowed to evolve in parallel under each of the following conditions (Fig. 1): selective pressure with ceftazidime (populations 1 to 4), selective pressure with ceftazidime-avibactam (populations 5 to 8), and in the absence of any selective pressure (populations 9 to 12). The susceptibility of each population to the selecting antibiotic was determined every 5 days by MIC test strip. However, after 20 days of

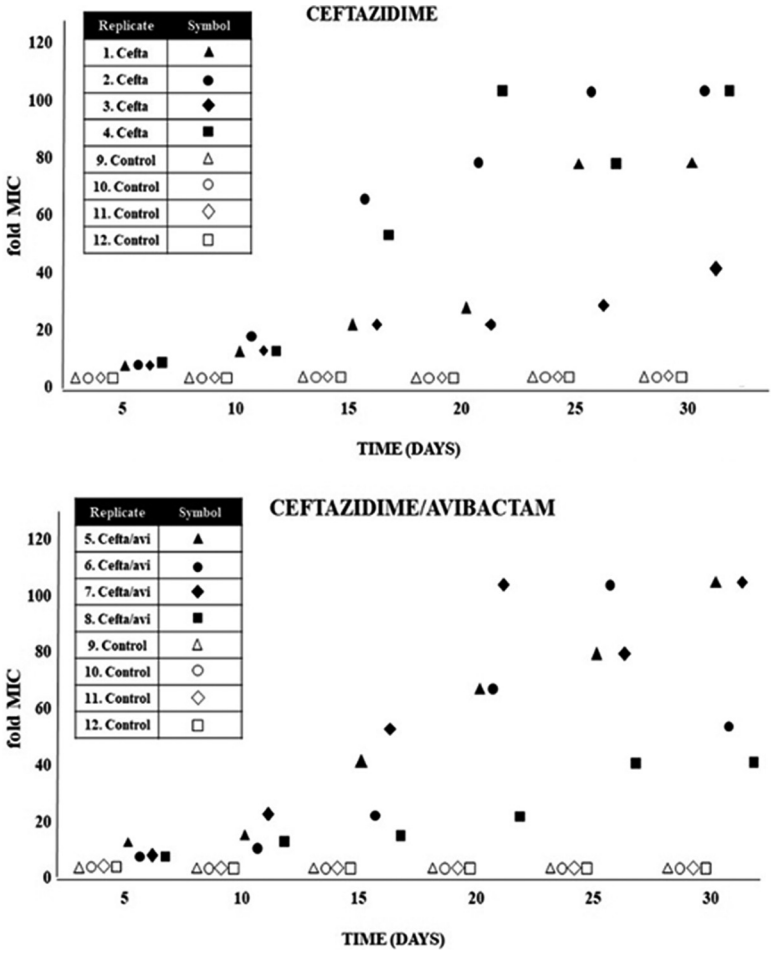

FIG 2 Evolution of *P. aeruginosa* PA14 under ceftazidime and ceftazidime-avibactam selective pressure. Graphs show the increase of the MICs over the evolution period ($MIC_{population\ X}/MIC_{PA14}$, where $MIC_{PA14} = 4\ \mu g/ml$ and X = 1 to 12) from the beginning of the experiment to high levels of resistance (doubling the antibiotic concentration every 5 days). The values were obtained by liquid MIC determination in a 96-well plate (see Table S3 in the supplemental material), because the detection limit of the ceftazidime MIC test strip is 256 $\mu g/ml$, limiting the assessment of resistance levels from day 20 to the end of the experiment.

evolution, MICs reached the highest limits of the MIC test strips and MICs were again determined for each evolutionary step, using a range of antibiotic concentrations (see Table S3 in the supplemental material). Stepwise evolutionary trajectories, in which the selected populations reached quite similar levels of resistance, were observed for both treatments (Fig. 2). These results suggest that avibactam inhibition may not be a guarantee of impeding *P. aeruginosa* from acquiring high-level ceftazidime resistance. An increase in the MIC of an antibiotic after experimental evolution does not necessarily imply that antibiotic-resistant mutants have been selected for; resistance may have arisen due to a phenotypic (inducible) adaptation to the presence of ceftazidime rather than to mutations (18–20). To address this possibility, the evolved populations were cultured in the absence of selection pressure (three sequential passages in Luria-Bertani [LB] broth), and the MICs were again determined. These were found not to vary, indicating that the observed modifications were due to the selection of stable mutants.

Cross-resistance and collateral sensitivity of the evolved populations. Taking into consideration the few therapeutic options for patients submitted to ceftazidime-avibactam therapy, knowing whether or not acquisition of resistance to this combination might alter susceptibility to other antibiotics is of crucial importance. To that end, susceptibility to a range of representative antibiotics was tested by disk diffusion assay

TABLE 1 MICs of antibiotics of different structural families in the populations evolved in the presence of either cefatzidime or ceftazidime-avibactam

Replicate[a]	MIC (μg/ml) for[b]:											
	TGC	TET	ATM	IPM	MEM	CAZ	CZA	NOR	AMK	CHL	ERY	FOF
PA14	2	12	1.5	0.5	0.19	1	1	0.25	1.5	24	32	16
1	2	32	≥256	8	32	≥256	≥256	1.5	1	≥256	≥256	4
2	1.5	24	≥256	4	32	≥256	≥256	1	0.5	≥256	≥256	1.5
3	2	16	≥256	2	32	≥256	≥256	1	0.75	≥256	≥256	4
4	2	16	≥256	3	32	≥256	≥256	1	0.5	≥256	≥256	2
5	0.75	4	≥256	32	32	≥256	≥256	0.25	0.75	≥256	≥256	3
6	0.5	4	≥256	3	16	≥256	≥256	0.25	1	≥256	≥256	4
7	0.38	4	96	32	32	≥256	≥256	0.25	2	≥256	≥256	6
8	0.75	8	≥256	4	32	≥256	≥256	0.75	0.75	≥256	≥256	4
9	3	12	1.5	0.75	0.25	1	1	0.25	1.5	24	32	24
10	3	12	1.5	0.5	0.19	1	1	0.25	1.5	24	32	24
11	3	12	1	0.75	0.25	1	1	0.25	1.5	24	32	16
12	3	16	1	0.75	0.25	1	1	0.38	1.5	24	32	12

[a]Populations were challenged as follows: 1 to 4, ceftazidime; 5 to 8, ceftazidime-avibactam; and 9 to 12, controls.
[b]TGC, tigecycline; TET, tetracycline; ATM, aztreonam; CAZ, ceftazidime; CZA, ceftazidime-avibactam; IPM, imipenem; MEM, meropenem; NOR, norfloxacin; AMK, amikacin; CHL, chloramphenicol; ERY, erythromycin; FOF, fosfomycin.

(see Table S4 in the supplemental material). From these results, a set of antibiotics was chosen for determining their MICs against the different evolved populations. Every evolved replicate showed altered susceptibility to antimicrobials belonging to different structural families (Table 1), implying that at least some resistance mutations were not ceftazidime or ceftazidime-avibactam specific. All populations evolved in the presence of either ceftazidime or ceftazidime-avibactam presented decreased susceptibility to other β-lactams, chloramphenicol, and erythromycin, and they were more susceptible to fosfomycin and amikacin. Notably, while populations evolved in the presence of ceftazidime were less susceptible to tetracycline and did not present changes in susceptibility to tigecycline, populations evolved in the presence of ceftazidime-avibactam were hypersusceptible to both antibiotics.

Analysis of mutations associated with the acquisition of resistance. To know the genetic events associated with the acquisition of resistance in the evolved populations, the genomes of each population, as well as that of the original PA14 strain, were sequenced on the last day of the experiment. Table 2 encompasses the resulting mutated genes and their functional significance, whereas Table S1 in the supplemental material shows the locations of all 40 genetic changes that were unveiled and were not present in control populations evolving in the absence of antibiotics. A total of 37 single-nucleotide variants (SNVs) and 3 multinucleotide variants (MNVs; deletions and substitutions of various nucleotides) were found, 36 located in genes and 4 in intergenic regions. Most mutations located in genes resulted in amino acid alterations, stop codons, or frameshifts. In addition, all of the populations evolved in the presence of antibiotics contained large chromosomal deletions (55 to 443 kbp), representing from 0.88% to 7.09% of the *P. aeruginosa* PA14 genome. Five different deletions were selected; all presented a 55-kbp common region (Fig. 3).

To verify the presence and the order of appearance of the genetic changes identified by WGS, the regions holding these mutations were amplified using specific oligonucleotides (see Table S2 in the supplemental material), and the amplicons were Sanger sequenced in each evolutionary step (Fig. 4). Regarding the large chromosomal deletions, primers located at the flanking sequences were used to verify their presence. In all cases, these analyses confirmed the information obtained from WGS.

Common mutations selected under either ceftazidime or ceftazidime-avibactam selection pressure. After 1 day of experimental evolution, all *P. aeruginosa* PA14 cultures challenged with antibiotic produced a brown pigment (Fig. 3) that appeared to be pyomelanin, the accumulation of which is normally due to the lack of homogentisate 1,2-dioxygenase activity provided by the enzyme HmgA (21). All of the chromosomal large deletions selected during evolution presented *hmgA* (Fig. 3). In addition,

TABLE 2 Mutated genes in ceftazidime- and ceftazidime-avibactam-evolved populations[a]

Treatment (populations)	Gene name	Functional classification	Replicate(s)	Previously described effect(s) on antibiotic susceptibility	Reference(s) or source
Both treatments	*nalD*	Efflux regulation	4, 6, 8	Quinolones, macrolides, tetracyclines, chloramphenicol, and β-lactams	24–26
	mexB	Efflux component	1, 3, 6, 8	Quinolones, macrolides, tetracyclines, chloramphenicol, and β-lactams	23
	ftsI	Penicillin binding protein	1, 5, 8	β-Lactams	27–30
	clpA	Protease activity	1, 5	Aztreonam	27
Ceftazidime (1–4)	*mexR*	Efflux regulation	1, 3	Quinolones, macrolides, tetracyclines, chloramphenicol, and β-lactams	23
	Upstream *mexA*	Efflux regulation	2	Quinolones, macrolides, tetracyclines, chloramphenicol, and β-lactams	23
	mpl	Peptidoglycan metabolism (β-lactamase)	2, 4	β-Lactams	22, 65
	orfN	Flagellin glycolysation	2	Ciprofloxacin, tigecycline, and tobramycin	34 and F. Sanz-García et al. (submitted for publication)
	infB	Translation factor	2		
	pitA	Phosphate transport	2, 4		
	grpE	Heat shock protein	3		
	clpP	Protease activity	3		
	dnaK	Chaperone	3	Several antibiotics	35
	dacB	Penicillin binding protein	4	β-Lactams	32
Ceftazidime-avibactam (5–8)	*dnaJ*	Chaperone	5	Triclosan	35
	pepA	Protease activity	6	Meropenem and aztreonam	27, 40
	ctpA	Protease activity	6		
	glnD	N_2 metabolism	6	Aminoglycosides and cephradine	35
	flgF	Flagellar component	6	Cephradine, cefoxitin, and chloramphenicol	35
	pcm	Protein repair/degradation	8		
	spoT	Stringent response	8	Piperacillin	41
	PA14_45870	TCS sensor	5	Carbapenems	36, 37
	PA14_45880	TCS regulator	7	Carbapenems	36, 37
	PA14_45890	Efflux component	5, 7	Carbapenems	36, 37

[a]The table shows previously described effects in the susceptibility to antibiotics of mutations in the genes that appear to be mutated in the experimental evolution.

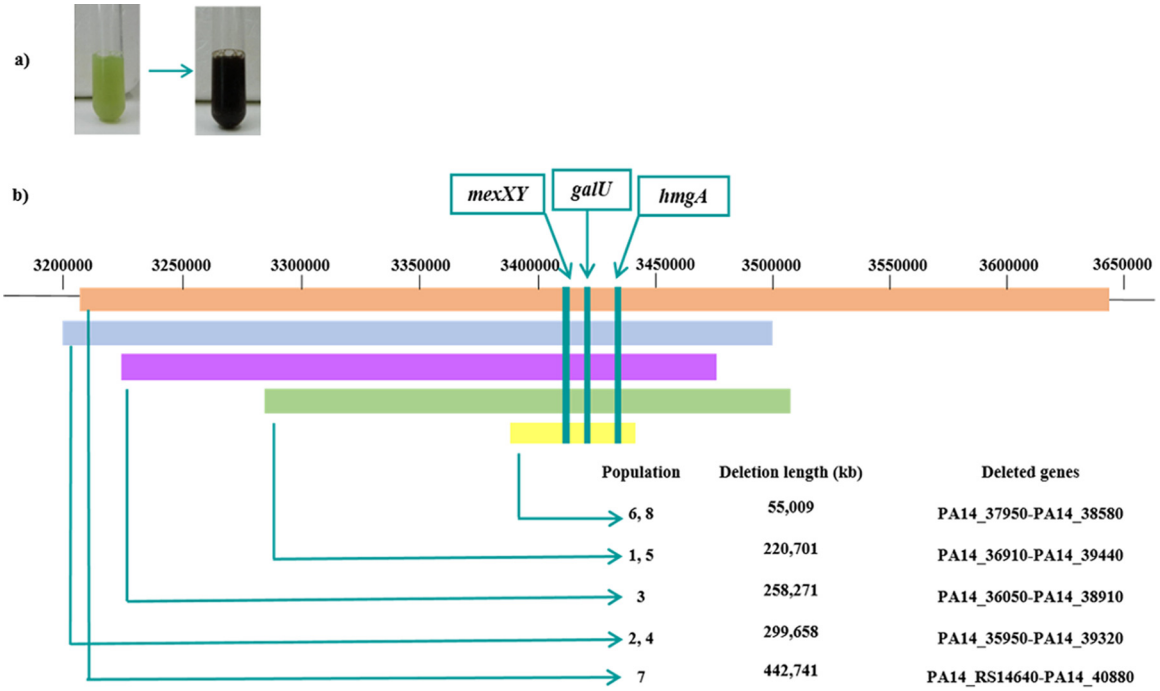

FIG 3 Large deletions present in all ceftazidime- and ceftazidime-avibactam-evolved populations since the first day of the experimental evolution. (a) A first event in the evolution in the presence of the antibiotics was an increase in pyomelanin accumulation due to the loss of *hmgA*, as the consequence of the deletion of large regions of *P. aeruginosa* genome. (b) The length of the deletions and the deleted genes in each replicate, as well as their genome localization, which corresponds with the *P. aeruginosa* UCBPP-PA14 reference chromosome (GenBank accession no. NC_008463).

the deletions also included *galU* (involved in lipopolysaccharide [LPS] biosynthesis), the inactivation of which reduces ceftazidime susceptibility (22), and the MDR efflux pump *mexXY-oprM*, which contributes to aminoglycoside resistance in *P. aeruginosa* (23). Deletion of the latter is likely the cause of the observed amikacin hypersusceptibility of all but one of the evolved populations (Table 1).

Another common element in both evolutions is *nalD*, which encodes a secondary repressor of MexAB-OprM (24, 25). Three out of eight replicates showed the same T11N change, which has been previously found in extensively drug-resistant (XDR) *P. aeruginosa* high-risk clones (26) that overexpress MexAB-OprM. Notably, four replicates (including the three presenting mutations in *nalD*) also presented mutations in *mexB*, indicating this efflux system to be a relevant element in the acquisition of resistance. Two other elements that are selected for in both treatments are *ftsI* and *clpA*. The first encodes PBP3, the target of different β-lactams (27, 28), which has been already found to be mutated in numerous resistant *P. aeruginosa* isolates. Indeed, the mutations R504C/H found in populations 1 and 5 are also present among isolates from widespread nosocomial *P. aeruginosa* clones (28–30). *clpA* encodes an intracellular protease involved in different aspects of *P. aeruginosa* physiology, in addition to aztreonam resistance (27, 31).

Mutations selected by ceftazidime. In addition to the observed mutations in *nalD*, which would allow *mexAB-oprM* overexpression, we also found mutations that should lead to the overexpression of this system in the populations evolving under ceftazidime challenge. Two populations carried mutants in *mexR*, which encodes a local repressor of *mexAB-oprM* expression. Another population presented a mutation upstream of *mexA* that might prevent the interaction of NalD with its operator (24), thus allowing *mexAB-oprM* overexpression.

Other mutations specifically selected for by ceftazidime were found in *mpl* and *dacB*. The proteins encoded by these genes are involved in the recycling of peptidoglycan muropeptides. In addition, they control the activity of AmpR and consequently the level

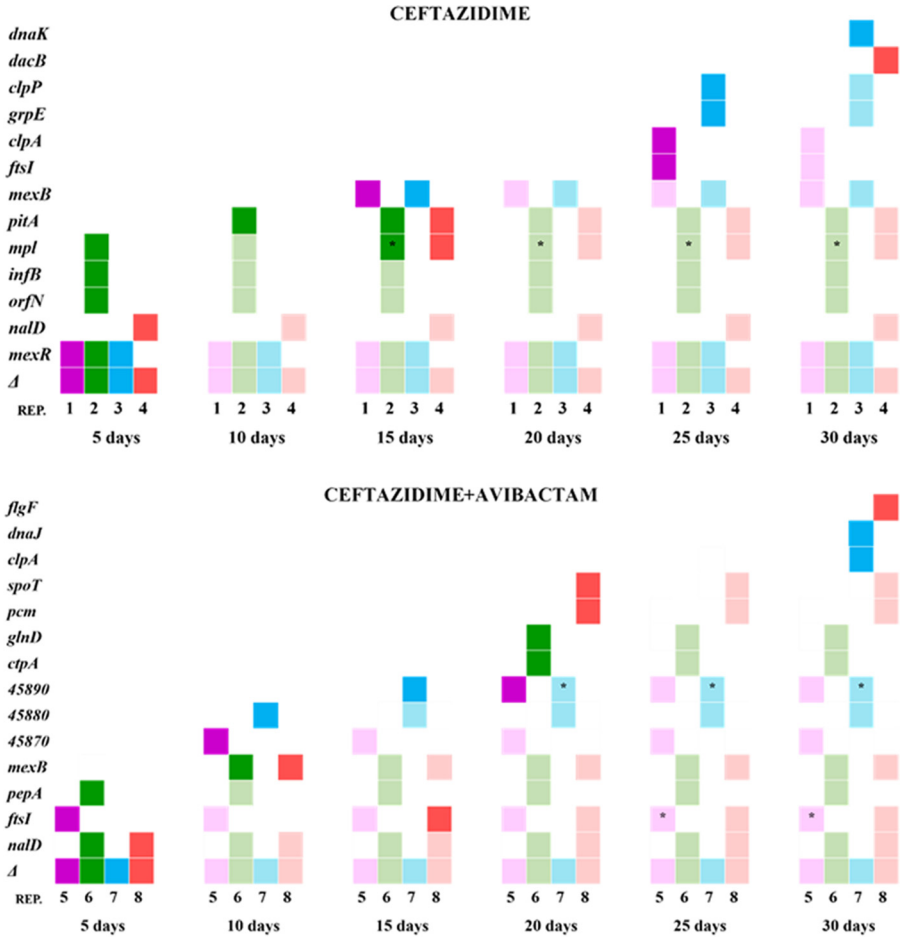

FIG 4 Order of appearance of genetic changes. The appearance of ceftazidime and ceftazidime-avibactam resistance mutations during the evolution process, as determined by PCR amplifications of known SNVs/MNVs in evolved populations. The *mexR* mutation in ceftazidime population 2 actually indicates that this mutation occurred in the intergenic region between *mexR* and *mexA*. An asterisk (*) refers to a second mutation in a gene that mutated previously. We cannot exclude the possibility that other mutations may have appeared over the 30-day evolution process. Δ, large deletion; Rep, replicate; intense-color square, the mutation appeared in this evolutionary step; light-color square, the mutation appeared in a previous evolutionary step.

of AmpC expression (22, 32), which is known to be a main element in *P. aeruginosa* resistance to β-lactams. Interestingly, *mpl* V124G (replicate 2; see Table S1 in the supplemental material) has been found before in a clinical isolate (*P. aeruginosa* NCGM1984). These findings, along with the aforementioned *ftsI* and *nalD* mutations, validate our experimental evolution approach as a valuable predictive model for the *in vivo* selection of antibiotic resistance.

Finally, mutations at *orfN*, *pitA*, *infB*, *grpE*, *clpP*, and *dnaK* were selected for in populations challenged with ceftazidime. *orfN* codes for a putative glycosyl transferase of type A flagellins (33). Mutations on this gene have been found in ciprofloxacin-resistant *P. aeruginosa* strains (34), and also in *P. aeruginosa* populations submitted to tigecycline and tobramycin experimental evolutions (F. Sanz-García, S. Hernando-Amado, and J. L. Martinez, submitted for publication). *pitA* encodes a phosphate transporter, *infB* encodes the translation initiation factor IF-2, and *dnaK*, *grpE*, and *clpP* encode proteins involved in regulatory gene networks involved in response to stress. None of these genes has been previously related to ceftazidime resistance, except for *dnaK*, the inactivation of which leads to stronger susceptibility to various antimicrobials in *Escherichia coli* (35).

Mutations selected by ceftazidime-avibactam. The challenge with ceftazidime-avibactam selected mutants in a predicted efflux pump (*PA14_45890* and *PA14_45910*), as well as in the two-component system (TCS) encoded by the operon *PA14_45870* and *PA14_45880*, likely regulating its expression. Previous studies have shown this efflux pump to be involved in *P. aeruginosa* intrinsic resistance (36) and susceptibility to carbapenems (37). Regarding the substrate recognition profile this pump might display, it is remarkable that populations 5 and 7, which present the aforementioned mutations, show a much lower susceptibility to imipenem than any other replicate (Table 1), suggesting this pump to have certain specificity to carbapenems.

Other mutations that were selected upon ceftazidime-avibactam treatment were found in *pepA*, *spoT*, *dnaJ*, and *flgF*. *pepA* encodes a protease necessary for *P. aeruginosa* cytotoxicity, virulence, and, consequently, lung infection (38, 39). Although its implication in antibiotic resistance has not been studied in detail, it has been reported that its inactivation confers meropenem resistance in *P. aeruginosa* (40). Moreover, *pepA* mutants are selected in the presence of aztreonam (27). SpoT has been related to piperacillin resistance (41), while DnaJ, a chaperone protein, and FlgF, a flagellar basal body rod protein (42), have been reported to modify the susceptibility of *E. coli* to a range of antibiotics when they are inactivated (35).

The other mutations that were selected for in populations under ceftazidime-avibactam challenge, namely those occurring in *ctpA*, an essential gene for the transition between acute and chronic *P. aeruginosa* infection (43), *pcm*, which encodes a L-isoaspartate carboxyl methyltransferase type II that participates in protein repair and degradation, and *glnD*, which is implicated in N_2 metabolism (44), have not been reported to be involved in antibiotic resistance.

Determination of the β-lactamase activity of the evolved populations. The evolution of ceftazidime resistance has been previously investigated in a *P. aeruginosa* PAO1 background (45), and resistance was mainly driven by combinations of mutations leading to greatly enhanced AmpC expression and ceftazidime resistance, a feature that was not so clear in the case of *P. aeruginosa* PA14, an strain in which the level of β-lactamase expression of *ampD* and *dacB* mutants is lower in comparison (46).To ascertain whether or not the overexpression of AmpC is a general outcome in our evolution, the β-lactamase activity of the evolved populations was measured at the endpoint of the experiment. As shown in Fig. 5, populations 2 and 4, which harbor *mpl* and *dacB* mutations, presented a large increase in β-lactamase production. The other populations evolved in the presence of ceftazidime and those evolved in the presence of ceftazidime-avibactam, which do not carry these mutations, also presented an increase in β-lactamase production, although the level reached was lower in comparison.

DISCUSSION

The use of β-lactamase inhibitors has reemerged as a fruitful strategy for fighting infections by MDR bacteria. Among them, ceftazidime-avibactam can be a useful combination for treating infections by different organisms, including *P. aeruginosa*. The analysis of the mechanisms of resistance to previous β-lactam/β-lactamase inhibitor combinations, such as amoxicillin-clavulanate, have shown that the main mechanisms selected by their use have been increased expression or mutation of preexisting β-lactamases and acquisition of new ones by HGT (47–51). *P. aeruginosa* has already acquired different carbapenemases that might be important elements in ceftazidime-avibactam resistance. In addition, resistance can be achieved through mutations, particularly in the case of *P. aeruginosa* causing chronic infections. To identify potential mutations involved in the acquisition of either ceftazidime or ceftazidime-avibactam resistance, bacterial populations were submitted to increasing selective concentrations of these antimicrobials. In both cases, the first event in the evolution seems to be the deletion of large regions of the *P. aeruginosa* chromosome that comprise, among several other genes, *hmgA*, *galU*, and *mexXY*. A similar situation has been previously reported in other *P. aeruginosa* experimental evolution assays in the presence of

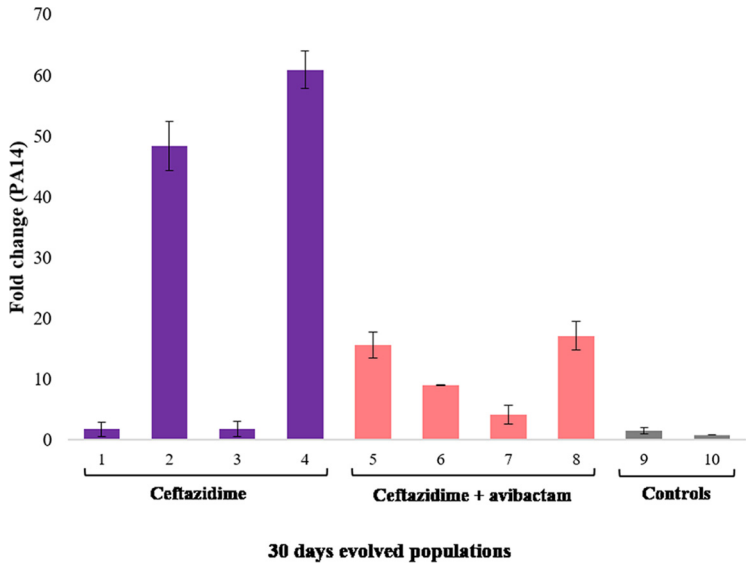

FIG 5 β-Lactamase activity in final evolved populations. The figure shows the relative amount of β-lactamase activity in 30-day ceftazidime- and ceftazidime-avibactam-evolved populations, as well as the activities of two replicates evolved in the absence of antibiotics. Fold changes were estimated with respect to the value given by the *P. aeruginosa* PA14 strain. Error bars indicate standard deviations of the results from two independent experiments.

β-lactams, such as piperacillin (41) and meropenem (45). Additionally, pyomelanin-producing mutants are regularly isolated from infections; up to 13% of CF patients harbor pyomelanin-producing mutants (52), likely because the production of pyomelanin increases resistance to oxidative stress and persistence in chronic lung infections (21). Recent work has also shown that these mutations can be selected to prevent bacteriophage predation (53). Notably, melanogenic clinical isolates of *P. aeruginosa* present large chromosomal deletions similar to those reported in the present work (54). Our results then support that ceftazidime may select for these genome deletions, and the presence of avibactam cannot prevent them from happening. It might be possible that deletions are the consequence of increased recombination triggered by the presence of the antibiotic. However, the fact that *P. aeruginosa* PA14 evolved in the presence of ciprofloxacin does not produce pyomelanin (a marker of these deletions) and that pyomelanin-producing mutants are selected when a *P. aeruginosa recA*-defective strain is challenged with either ceftazidime or ceftazidime-avibactam (data not shown), goes against this possibility. Besides the already known effect of the lack of *galU* on susceptibility to β-lactams, the absence of other genes located in the deletion, such as *mexXY*, may affect *P. aeruginosa* susceptibility to antibiotics. Deletion of this pump is likely the cause of the observed hypersusceptibility to amikacin of the evolved populations. In addition, it might have an indirect effect on the decreased susceptibility to β-lactams, particularly in the case of those strains carrying mutations in the repressors of *mexAB-oprM*. MexAB-OprM is an important determinant of intrinsic *P. aeruginosa* resistance to different antibiotics, including to β-lactams (23). Furthermore, mutants overexpressing this efflux pump are regularly isolated from infections, and its expression has been shown to be prevalent among resistant *P. aeruginosa* clinical isolates (55–58). MexAB and MexXY share the outer membrane protein OprM, which produces antagonistic interactions when both systems are expressed (45, 59). Hence, MexXY-OprM elimination might favor the efficiency of β-lactam efflux, reducing the competition of both efflux pumps for OprM. Besides aminoglycoside hypersusceptibility, the selected mutants also present fosfomycin collateral susceptibility, although the causes of this phenotype are unknown. It has been shown that mutants defective in the *P. aeruginosa* peptidoglycan recycling pathway show a marked increase in fosfomycin susceptibility (60). Although mutations in these elements were not found in

the evolved populations, it is still possible that they might present altered levels of expression, an issue that remains to be explored.

Important elements in the acquisition of ceftazidime resistance include efflux pumps, particularly MexAB-OprM, since mutations in either the elements regulating its expression or in the efflux pump itself were found in six out of eight evolved populations, whereas the two remaining populations harbored mutants in the putative *PA14_45890* and *PA14_45910* operon and in its potential TCS regulator. While the substrates of MexAB-OprM are known and include β-lactams, the substrates of *PA14_4590* and *PA14_45910* are unknown. Nevertheless, it is remarkable that populations presenting mutations on this determinant display a much lower susceptibility to imipenem than does any other replicate, suggesting this pump to have certain specificity to β-lactams, a feature that deserves further work.

Mutations in elements involved in the regulation of AmpC expression were selected when just ceftazidime was used for selection and not in the presence of ceftazidime-avibactam. This suggests that, at least in the *P. aeruginosa* PA14 background, the efficient inhibition by avibactam of intrinsic β-lactamases precludes the emergence of mechanisms based on their overexpression, and other mechanisms, including the above-mentioned large deletions and modifications in the activity of efflux pumps, are preferentially selected. This does not necessarily mean that resistance to ceftazidime-avibactam cannot be associated with changes in the activity of AmpC, particularly if the challenged isolate is already resistant to ceftazidime. Indeed, avibactam-resistant mutants presenting changes in the avibactam binding pocket of AmpC are selected *in vitro* at low frequency from AmpC-overexpressing ceftazidime-resistant *P. aeruginosa* isolates, and the role of mutations in AmpR, the regulator of AmpC expression, in developing ceftazidime-avibactam resistance has been recently explored (61).

Although most of the mutants reported here have been previously associated with antibiotic resistance, it is still possible that some of the mutations might be selected for compensating the fitness costs associated with the acquisition of resistance. This might be the case of *ctpA*, *pcm*, or the mutations at structural elements of efflux pumps that were selected after mutations in the regulators of their expression. For the last, it might also be possible that these mutations increase the capability to extrude the antibiotic substrates, as described for AcrB (62). The fact that in all evolved populations, mutants in efflux pumps are selected, provides an explanation of the cross-resistance phenotype observed in all resistant strains. This situation might be of concern, since both ceftazidime and ceftazidime-avibactam might select for resistance to other antibiotics, at least in chronic infections in which mutation is the main cause of acquisition of resistance.

P. aeruginosa evolution in chronic infections frequently involves large genome deletions (63), which are usually linked to the production of pyomelanin (54). Whether these deletions are selected by antibiotic treatment or are just the consequence of the adaptation to the environment of the lungs of the CF patient remains to be established. However, this evolution provides a link between antibiotic resistance and virulence for this relevant pathogen. In any case, and given that deletions containing *galU* and *hmgA* appear to be a first step in the evolution toward ceftazidime-avibactam resistance, pyomelanin production could be considered a marker in the selection of the antibiotic of choice for treating *P. aeruginosa* infections. Both *in vitro* work, including the results here shown, and the analysis of clinical pyomelanin producers have shown that these isolates are usually hypersusceptible to aminoglycosides, probably because the deletions they present include *mexXY*. It would then be judicious to use aminoglycosides and not β-lactams for treating infections by pyomelanin-producing *P. aeruginosa* strains.

MATERIALS AND METHODS

Growth conditions and antibiotic susceptibility assays. Unless otherwise stated, bacteria were grown in Luria Bertani (LB) broth at 37°C with shaking at 250 rpm. The susceptibility to tigecycline, tetracycline, aztreonam, ceftazidime, imipenem, meropenem, ciprofloxacin, levofloxacin, norfloxacin, tobramycin, streptomycin, amikacin, gentamicin, colistin, polymyxin B, chloramphenicol, fosfomycin, and

erythromycin was determined by disk diffusion in Mueller-Hinton agar (MHA) (Sigma) at 37°C. For a set of antibiotics, MICs were determined using MIC test strips (Liofilchem). MICs of ceftazidime and ceftazidime-avibactam were determined in LB broth by pouring into microtiter plates specific antibiotic concentrations in an arithmetic scale spanning from 0.75 to 800 μg/ml.

Experimental evolution procedure. Twelve bacterial populations from a stock *P. aeruginosa* PA14 culture (four controls without antibiotic, four populations challenged with ceftazidime, and four populations challenged with ceftazidime-avibactam) were grown in parallel in LB broth for 30 consecutive days. Each day, the cultures were diluted (1/250) in fresh LB broth. The concentrations of ceftazidime used for selection were increased over the evolution experiment from the concentration that hinders the growth of *P. aeruginosa* PA14 under these culture conditions (4 μg/ml) up to 128 μg/ml, doubling them every 5 days. The avibactam concentration was maintained at a constant 4 μg/ml, as used in clinical tests (64). On some occasions, the cultures did not grow when antibiotic concentration was increased, in which case the selection was kept at the concentration that allowed growth. Every 5 days, samples from each culture were preserved at −80°C for further research.

Whole-genome sequencing (WGS). A Gnome DNA kit (MP Biomedicals) was used to extract genomic DNA. WGS was performed by Sistemas Genómicos S.L. The quality of the extracted material was analyzed via a 4200 TapeStation high-sensitivity assay, and the DNA concentration was ascertained by real-time PCR using a LightCycler 480 device (Roche). Libraries were obtained without amplification, following Illumina protocols, and were pair end sequenced (100 × 2) in an Illumina HiSeq 2500 sequencer. The average number of reads per sample was 7,178,870, which represents a 200× coverage, on average.

Bioinformatics analysis of WGS and confirmation of genetic changes. Mutations in the evolved populations were identified using CLC Genomics Workbench 9.0 (Qiagen). *P. aeruginosa* UCBPP-PA14 reference chromosome (GenBank accession number NC_008463) was used to align the reads obtained from WGS data (previously trimmed). Sanger sequencing was used to verify and to settle the order of appearance of the putative mutations found via WGS (Table S1). Thirty-two pairs of primers, which amplified 200- to 400-bp regions containing each genetic modification, were designed (see Table S2 in the supplemental material). After PCR amplification, the corresponding amplicons were purified using the QIAquick PCR purification kit (Qiagen) and sequenced at GATC Biotech.

Determination of β-lactamase activity. Cells were grown overnight at 37°C and 250 rpm in 20 ml of LB broth. Afterwards, they were harvested by centrifugation (7,000 rpm for 10 min) and resuspended in 500 μl of 0.1 M Na_2HPO_4 (pH 7.4) buffer. Crude protein extracts were prepared by sonication on ice (0.7 Hz) and centrifuged again (13,000 rpm, 15 min). The protein content of each extract was determined using the Bradford protein assay with bovine serum albumin as a standard. The β-lactamase activity was quantified spectrophotometrically by measuring the change in absorbance at 486 nm, using the chromogenic β-lactamase substrate nitrocefin at 500 μg/ml (Oxoid, Basingstoke, United Kingdom) and 0.1 M Na_2HPO_4 (pH 7.4) as the test buffer. The assay was performed using the Infinite M200 plate reader (TECAN) for 2 h at 37°C, with measurements every 2 min.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01379-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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REFERENCES

1. Silby MW, Winstanley C, Godfrey SA, Levy SB, Jackson RW. 2011. *Pseudomonas* genomes: diverse and adaptable. FEMS Microbiol Rev 35:652–680. https://doi.org/10.1111/j.1574-6976.2011.00269.x.
2. Martinez-Solano L, Macia MD, Fajardo A, Oliver A, Martinez JL. 2008. Chronic *Pseudomonas aeruginosa* infection in chronic obstructive pulmonary disease. Clin Infect Dis 47:1526–1533. https://doi.org/10.1086/593186.
3. Talwalkar JS, Murray TS. 2016. The approach to *Pseudomonas aeruginosa* in cystic fibrosis. Clin Chest Med 37:69–81. https://doi.org/10.1016/j.ccm.2015.10.004.
4. Vila J, Martínez JL. 2008. Clinical impact of the over-expression of efflux pump in nonfermentative Gram-negative bacilli, development of efflux pump inhibitors. Current drug targets 9:797–807. https://doi.org/10.2174/138945008785747806.
5. Oliver A, Mulet X, Lopez-Causape C, Juan C. 2015. The increasing threat of *Pseudomonas aeruginosa* high-risk clones. Drug Resist Updat 21–22:41–59. https://doi.org/10.1016/j.drup.2015.08.002.
6. Tzouvelekis LS, Markogiannakis A, Psichogiou M, Tassios PT, Daikos GL. 2012. Carbapenemases in *Klebsiella pneumoniae* and other *Enterobacteriaceae*: an evolving crisis of global dimensions. Clin Microbiol Rev 25:682–707. https://doi.org/10.1128/CMR.05035-11.
7. Woodford N, Turton JF, Livermore DM. 2011. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol Rev 35:736–755. https://doi.org/10.1111/j.1574-6976.2011.00268.x.
8. Buynak JD. 2006. Understanding the longevity of the beta-lactam antibiotics and of antibiotic/beta-lactamase inhibitor combinations. Biochem Pharmacol 71:930–940. https://doi.org/10.1016/j.bcp.2005.11.012.

9. Liscio JL, Mahoney MV, Hirsch EB. 2015. Ceftolozane/tazobactam and ceftazidime/avibactam: two novel beta-lactam/beta-lactamase inhibitor combination agents for the treatment of resistant Gram-negative bacterial infections. Int J Antimicrob Agents 46:266–271. https://doi.org/10.1016/j.ijantimicag.2015.05.003.
10. Coleman K. 2011. Diazabicyclooctanes (DBOs): a potent new class of non-beta-lactam beta-lactamase inhibitors. Curr Opin Microbiol 14:550–555. https://doi.org/10.1016/j.mib.2011.07.026.
11. Aktas Z, Kayacan C, Oncul O. 2012. In vitro activity of avibactam (NXL104) in combination with beta-lactams against Gram-negative bacteria, including OXA-48 beta-lactamase-producing *Klebsiella pneumoniae*. Int J Antimicrob Agents 39:86–89. https://doi.org/10.1016/j.ijantimicag.2011.09.012.
12. Hayes MV, Orr DC. 1983. Mode of action of ceftazidime: affinity for the penicillin-binding proteins of *Escherichia coli* K12, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. J Antimicrob Chemother 12:119–126. https://doi.org/10.1093/jac/12.2.119.
13. Hidalgo JA, Vinluan CM, Antony N. 2016. Ceftazidime/avibactam: a novel cephalosporin/nonbeta-lactam beta-lactamase inhibitor for the treatment of complicated urinary tract infections and complicated intra-abdominal infections. Drug Des Devel Ther 10:2379–2386. https://doi.org/10.2147/DDDT.S110946.
14. Pitart C, Marco F, Keating TA, Nichols WW, Vila J. 2015. Activity of ceftazidime-avibactam against fluoroquinolone-resistant *Enterobacteriaceae* and *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 59:3059–3065. https://doi.org/10.1128/AAC.05136-14.
15. Calvopina K, Hinchliffe P, Brem J, Heesom KJ, Johnson S, Cain R, Lohans CT, Fishwick CWG, Schofield CJ, Spencer J, Avison MB. 2017. Structural/mechanistic insights into the efficacy of nonclassical beta-lactamase inhibitors against extensively drug resistant *Stenotrophomonas maltophilia* clinical isolates. Mol Microbiol 106:492–504. https://doi.org/10.1111/mmi.13831.
16. Lopez-Hernandez I, Alonso N, Fernandez-Martinez M, Zamorano L, Rivera A, Oliver A, Conejo MC, Martinez-Martinez L, Navarro F, Pascual A. 2017. Activity of ceftazidime-avibactam against multidrug-resistance *Enterobacteriaceae* expressing combined mechanisms of resistance. Enferm Infecc Microbiol Clin 35:499–504. https://doi.org/10.1016/j.eimc.2016.09.013.
17. Fraile-Ribot PA, Cabot G, Mulet X, Perianez L, Martin-Pena ML, Juan C, Perez JL, Oliver A. 2017. Mechanisms leading to *in vivo* ceftolozane/tazobactam resistance development during the treatment of infections caused by MDR *Pseudomonas aeruginosa*. J Antimicrob Chemother 73:658–663. https://doi.org/10.1093/jac/dkx424.
18. Levin BR, Rozen DE. 2006. Non-inherited antibiotic resistance. Nat Rev Microbiol 4:556–562. https://doi.org/10.1038/nrmicro1445.
19. Martinez JL, Rojo F. 2011. Metabolic regulation of antibiotic resistance. FEMS Microbiol Rev 35:768–789. https://doi.org/10.1111/j.1574-6976.2011.00282.x.
20. Martinez JL, Fajardo A, Garmendia L, Hernandez A, Linares JF, Martinez-Solano L, Sanchez MB. 2009. A global view of antibiotic resistance. FEMS Microbiol Rev 33:44–65. https://doi.org/10.1111/j.1574-6976.2008.00142.x.
21. Rodriguez-Rojas A, Mena A, Martin S, Borrell N, Oliver A, Blazquez J. 2009. Inactivation of the *hmgA* gene of *Pseudomonas aeruginosa* leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection. Microbiology 155:1050–1057. https://doi.org/10.1099/mic.0.024745-0.
22. Alvarez-Ortega C, Wiegand I, Olivares J, Hancock RE, Martinez JL. 2010. Genetic determinants involved in the susceptibility of *Pseudomonas aeruginosa* to beta-lactam antibiotics. Antimicrob Agents Chemother 54:4159–4167. https://doi.org/10.1128/AAC.00257-10.
23. Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 44:3322–3327. https://doi.org/10.1128/AAC.44.12.3322-3327.2000.
24. Morita Y, Cao L, Gould VC, Avison MB, Poole K. 2006. *nalD* encodes a second repressor of the *mexAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*. J Bacteriol 188:8649–8654. https://doi.org/10.1128/JB.01342-06.
25. Sobel ML, Hocquet D, Cao L, Plesiat P, Poole K. 2005. Mutations in PA3574 (nalD) lead to increased MexAB-OprM expression and multidrug resistance in laboratory and clinical isolates of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 49:1782–1786. https://doi.org/10.1128/AAC.49.5.1782-1786.2005.
26. Cabot G, Ocampo-Sosa AA, Dominguez MA, Gago JF, Juan C, Tubau F, Rodriguez C, Moya B, Pena C, Martinez-Martinez L, Oliver A, Spanish Network for Research in Infectious D. 2012. Genetic markers of widespread extensively drug-resistant *Pseudomonas aeruginosa* high-risk clones. Antimicrob Agents Chemother 56:6349–6357. https://doi.org/10.1128/AAC.01388-12.
27. Jorth P, McLean K, Ratjen A, Secor PR, Bautista GE, Ravishankar S, Rezayat A, Garudathri J, Harrison JJ, Harwood RA, Penewit K, Waalkes A, Singh PK, Salipante SJ. 2017. Evolved aztreonam resistance is multifactorial and can produce hypervirulence in *Pseudomonas aeruginosa*. mBio 8:e00517-17. https://doi.org/10.1128/mBio.00517-17.
28. Cabot G, Lopez-Causape C, Ocampo-Sosa AA, Sommer LM, Dominguez MA, Zamorano L, Juan C, Tubau F, Rodriguez C, Moya B, Pena C, Martinez-Martinez L, Plesiat P, Oliver A. 2016. Deciphering the resistome of the widespread *Pseudomonas aeruginosa* sequence type 175 international high-risk clone through whole-genome sequencing. Antimicrob Agents Chemother 60:7415–7423. https://doi.org/10.1128/AAC.02676-15.
29. Lopez-Causape C, Sommer LM, Cabot G, Rubio R, Ocampo-Sosa AA, Johansen HK, Figuerola J, Canton R, Kidd TJ, Molin S, Oliver A. 2017. Evolution of the *Pseudomonas aeruginosa* mutational resistome in an international cystic fibrosis clone. Sci Rep 7:5555. https://doi.org/10.1038/s41598-017-05621-5.
30. Kos VN, Deraspe M, McLaughlin RE, Whiteaker JD, Roy PH, Alm RA, Corbeil J, Gardner H. 2015. The resistome of *Pseudomonas aeruginosa* in relationship to phenotypic susceptibility. Antimicrob Agents Chemother 59:427–436. https://doi.org/10.1128/AAC.03954-14.
31. Fernandez L, Breidenstein EB, Song D, Hancock RE. 2012. Role of intracellular proteases in the antibiotic resistance, motility, and biofilm formation of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 56:1128–1132. https://doi.org/10.1128/AAC.05336-11.
32. Aguilera Rossi CG, Gomez-Puertas P, Ayala Serrano JA. 2016. *In vivo* functional and molecular characterization of the penicillin-binding protein 4 (DacB) of *Pseudomonas aeruginosa*. BMC Microbiol 16:234. https://doi.org/10.1186/s12866-016-0853-x.
33. Schirm M, Arora SK, Verma A, Vinogradov E, Thibault P, Ramphal R, Logan SM. 2004. Structural and genetic characterization of glycosylation of type a flagellin in *Pseudomonas aeruginosa*. J Bacteriol 186:2523–2531. https://doi.org/10.1128/JB.186.9.2523-2531.2004.
34. Wong A, Rodrigue N, Kassen R. 2012. Genomics of adaptation during experimental evolution of the opportunistic pathogen *Pseudomonas aeruginosa*. PLoS Genet 8:e1002928. https://doi.org/10.1371/journal.pgen.1002928.
35. Liu A, Tran L, Becket E, Lee K, Chinn L, Park E, Tran K, Miller JH. 2010. Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout collection: generating an antibiotic bar code. Antimicrob Agents Chemother 54:1393–1403. https://doi.org/10.1128/AAC.00906-09.
36. Fajardo A, Martinez-Martin N, Mercadillo M, Galan JC, Ghysels B, Matthijs S, Cornelis P, Wiehlmann L, Tummler B, Baquero F, Martinez JL. 2008. The neglected intrinsic resistome of bacterial pathogens. PLoS One 3:e1619. https://doi.org/10.1371/journal.pone.0001619.
37. Kohler T, Michea-Hamzehpour M, Epp SF, Pechere JC. 1999. Carbapenem activities against *Pseudomonas aeruginosa*: respective contributions of OprD and efflux systems. Antimicrob Agents Chemother 43:424–427.
38. Hauser AR, Kang PJ, Engel JN. 1998. PepA, a secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence. Mol Microbiol 27:807–818. https://doi.org/10.1046/j.1365-2958.1998.00727.x.
39. Potvin E, Lehoux DE, Kukavica-Ibrulj I, Richard KL, Sanschagrin F, Lau GW, Levesque RC. 2003. *In vivo* functional genomics of *Pseudomonas aeruginosa* for high-throughput screening of new virulence factors and antibacterial targets. Environ Microbiol 5:1294–1308. https://doi.org/10.1046/j.1462-2920.2003.00542.x.
40. Isabella VM, Campbell AJ, Manchester J, Sylvester M, Nayar AS, Ferguson KE, Tommasi R, Miller AA. 2015. Toward the rational design of carbapenem uptake in *Pseudomonas aeruginosa*. Chem Biol 22:535–547. https://doi.org/10.1016/j.chembiol.2015.03.018.
41. Yen P, Papin JA. 2017. History of antibiotic adaptation influences microbial evolutionary dynamics during subsequent treatment. PLoS Biol 15:e2001586. https://doi.org/10.1371/journal.pbio.2001586.
42. Homma M, Kutsukake K, Hasebe M, Iino T, Macnab RM. 1990. FlgB, FlgC,

FlgF and FlgG. A family of structurally related proteins in the flagellar basal body of *Salmonella* typhimurium. J Mol Biol 211:465–477.
43. Seo J, Darwin AJ. 2013. The *Pseudomonas aeruginosa* periplasmic protease CtpA can affect systems that impact its ability to mount both acute and chronic infections. Infect Immun 81:4561–4570. https://doi.org/10.1128/IAI.01035-13.
44. Contreras A, Drummond M, Bali A, Blanco G, Garcia E, Bush G, Kennedy C, Merrick M. 1991. The product of the nitrogen fixation regulatory gene *nfrX* of *Azotobacter vinelandii* is functionally and structurally homologous to the uridylyltransferase encoded by *glnD* in enteric bacteria. J Bacteriol 173:7741–7749. https://doi.org/10.1128/jb.173.24.7741-7749.1991.
45. Cabot G, Zamorano L, Moya B, Juan C, Navas A, Blazquez J, Oliver A. 2016. Evolution of *Pseudomonas aeruginosa* antimicrobial resistance and fitness under low and high mutation rates. Antimicrob Agents Chemother 60:1767–1778. https://doi.org/10.1128/AAC.02676-15.
46. Zamorano L, Moya B, Juan C, Oliver A. 2010. Differential beta-lactam resistance response driven by *ampD* or *dacB* (PBP4) inactivation in genetically diverse *Pseudomonas aeruginosa* strains. J Antimicrob Chemother 65:1540–1542. https://doi.org/10.1093/jac/dkq142.
47. Reading C, Cole M. 1977. Clavulanic acid: a beta-lactamase-inhiting beta-lactam from *Streptomyces clavuligerus*. Antimicrob Agents Chemother 11:852–857. https://doi.org/10.1128/AAC.11.5.852.
48. Baquero F, Reig M. 1989. Mechanisms of antimicrobial resistance in anaerobic bacteria: the predictive approach. Scand J Infect Dis Suppl 62:25–28.
49. Blazquez J, Baquero MR, Canton R, Alos I, Baquero F. 1993. Characterization of a new TEM-type beta-lactamase resistant to clavulanate, sulbactam, and tazobactam in a clinical isolate of *Escherichia coli*. Antimicrob Agents Chemother 37:2059–2063. https://doi.org/10.1128/AAC.37.10.2059.
50. Canton R, Gonzalez-Alba JM, Galan JC. 2012. CTX-M enzymes: origin and diffusion. Front Microbiol 3:110. https://doi.org/10.3389/fmicb.2012.00110.
51. Toussaint KA, Gallagher JC. 2015. Beta-lactam/beta-lactamase inhibitor combinations: from then to now. Ann Pharmacother 49:86–98. https://doi.org/10.1177/1060028014556652.
52. Mayer-Hamblett N, Rosenfeld M, Gibson RL, Ramsey BW, Kulasekara HD, Retsch-Bogart GZ, Morgan W, Wolter DJ, Pope CE, Houston LS, Kulasekara BR, Khan U, Burns JL, Miller SI, Hoffman LR. 2014. *Pseudomonas aeruginosa in vitro* phenotypes distinguish cystic fibrosis infection stages and outcomes. Am J Respir Crit Care Med 190:289–297. https://doi.org/10.1164/rccm.201404-0681OC.
53. Shen M, Zhang H, Shen W, Zou Z, Lu S, Li G, He X, Agnello M, Shi W, Hu F, Le S. 2018. *Pseudomonas aeruginosa* MutL promotes large chromosomal deletions through non-homologous end joining to prevent bacteriophage predation. Nucleic Acids Res 46:4505–4514. https://doi.org/10.1093/nar/gky160.
54. Hocquet D, Petitjean M, Rohmer L, Valot B, Kulasekara HD, Bedel E, Bertrand X, Plesiat P, Kohler T, Pantel A, Jacobs MA, Hoffman LR, Miller SI. 2016. Pyomelanin-producing *Pseudomonas aeruginosa* selected during chronic infections have a large chromosomal deletion which confers resistance to pyocins. Environ Microbiol 18:3482–3493. https://doi.org/10.1111/1462-2920.13336.
55. Chalhoub H, Saenz Y, Rodriguez-Villalobos H, Denis O, Kahl BC, Tulkens PM, Van Bambeke F. 2016. High-level resistance to meropenem in clinical isolates of *Pseudomonas aeruginosa* in the absence of carbapenemases: role of active efflux and porin alterations. Int J Antimicrob Agents 48:740–743. https://doi.org/10.1016/j.ijantimicag.2016.09.012.
56. Pan YP, Xu YH, Wang ZX, Fang YP, Shen JL. 2016. Overexpression of MexAB-OprM efflux pump in carbapenem-resistant *Pseudomonas aeruginosa*. Arch Microbiol 198:565–571. https://doi.org/10.1007/s00203-016-1215-7.
57. Riou M, Avrain L, Carbonnelle S, El Garch F, Pirnay JP, De Vos D, Plesiat P, Tulkens PM, Van Bambeke F. 2016. Increase of efflux-mediated resistance in *Pseudomonas aeruginosa* during antibiotic treatment in patients suffering from nosocomial pneumonia. Int J Antimicrob Agents 47:77–83. https://doi.org/10.1016/j.ijantimicag.2015.11.004.
58. Castanheira M, Mills JC, Farrell DJ, Jones RN. 2014. Mutation-driven beta-lactam resistance mechanisms among contemporary ceftazidime-nonsusceptible *Pseudomonas aeruginosa* isolates from U.S. hospitals. Antimicrob Agents Chemotherapy 58:6844–6850. https://doi.org/10.1128/AAC.03681-14.
59. Mulet X, Moya B, Juan C, Macia MD, Perez JL, Blazquez J, Oliver A. 2011. Antagonistic interactions of *Pseudomonas aeruginosa* antibiotic resistance mechanisms in planktonic but not biofilm growth. Antimicrob Agents Chemother 55:4560–4568. https://doi.org/10.1128/AAC.00519-11.
60. Hamou-Segarra M, Zamorano L, Vadlamani G, Chu M, Sanchez-Diener I, Juan C, Blazquez J, Hattie M, Stubbs KA, Mark BL, Oliver A. 2017. Synergistic activity of fosfomycin, beta-lactams and peptidoglycan recycling inhibition against *Pseudomonas aeruginosa*. J Antimicrob Chemother 72:448–454. https://doi.org/10.1093/jac/dkw456.
61. Gifford DR, Furio V, Papkou A, Vogwill T, Oliver A, MacLean RC. 2018. Identifying and exploiting genes that potentiate the evolution of antibiotic resistance. Nat Ecol Evol 2:1033–1039. https://doi.org/10.1038/s41559-018-0547-x.
62. Blair JM, Bavro VN, Ricci V, Modi N, Cacciotto P, Kleinekathfer U, Ruggerone P, Vargiu AV, Baylay AJ, Smith HE, Brandon Y, Galloway D, Piddock LJ. 2015. AcrB drug-binding pocket substitution confers clinically relevant resistance and altered substrate specificity. Proc Natl Acad Sci U S A 112:3511–3516. https://doi.org/10.1073/pnas.1419939112.
63. Rau MH, Marvig RL, Ehrlich GD, Molin S, Jelsbak L. 2012. Deletion and acquisition of genomic content during early stage adaptation of *Pseudomonas aeruginosa* to a human host environment. Environ Microbiol 14:2200–2211. https://doi.org/10.1111/j.1462-2920.2012.02795.x.
64. Zhanel GG, Lawson CD, Adam H, Schweizer F, Zelenitsky S, Lagace-Wiens PR, Denisuik A, Rubinstein E, Gin AS, Hoban DJ, Lynch JP, III, Karlowsky JA. 2013. Ceftazidime-avibactam: a novel cephalosporin/beta-lactamase inhibitor combination. Drugs 73:159–177. https://doi.org/10.1007/s40265-013-0013-7.
65. Alvarez-Ortega C, Wiegand I, Olivares J, Hancock RE, Martinez JL. 2011. The intrinsic resistome of *Pseudomonas aeruginosa* to beta-lactams. Virulence 2:144–146. https://doi.org/10.4161/viru.2.2.15014.

Artículo VI

Rapid and robust evolution of collateral sensitivity in *Pseudomonas aeruginosa* antibiotic resistant mutants

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Entre los efectos que propicia la adquisición de resistencia a antibióticos, uno de ellos se ha postulado como posible baza con la que contender en la pugna contra dicha resistencia: la sensibilidad colateral a una droga nacida de la obtención de resistencia a otra. Desgraciadamente, el conocimiento sobre la reproducibilidad y conservación de este fenómeno en contextos genéticos dispares resalta por su parvedad, lo que pone en tela de juicio si esta estrategia podría ser agible. Sin embargo, en el Artículo V de esta tesis se observó un primer evento genético conservado en todas las poblaciones de *P. aeruginosa* PA14 evolucionadas en presencia de ceftazidima: la selección de grandes deleciones cromosómicas que provocan hiper-producción de piomelanina y, lo que es más importante, sensibilidad colateral a aminoglicósidos como la tobramicina. Este fenómeno también se documentó en publicaciones en las que se utilizaban otros β-lactámicos. Así, en este estudio se evaluó la robustez de este *trade-off* en una colección de mutantes resistentes a diversos antibióticos de *P. aeruginosa* PA14, sometidos a una evolución de corta duración en presencia de ceftazidima. El experimento confirmó la robustez de dicha sensibilidad colateral en la mayoría de contextos genéticos ensayados.

A la luz de este resultado, se propuso una posible explotación de esta sensibilidad colateral a tobramicina en la situación particular de una población piomelanogénica heterogénea de este patógeno, realidad factible en el pulmón de un paciente con FQ. Para ello, se llevó a cabo una evolución secuencial en presencia de tobramicina-ceftazidima de las poblaciones heterogéneas derivadas de cada uno de los fondos genéticos resistentes empleados. La primera fase con tobramicina erradicó la subpoblación piomelanogénica, resistente a ceftazidima e hipersensible a tobramicina, de la comunidad. A continuación, el viraje a ceftazidima acrecentó significativamente los niveles de sensibilidad a tobramicina, algo que de nuevo sobresalió por su robustez al darse en la mayoría de los contextos genéticos analizados: 23 de 27 poblaciones. Asimismo, las poblaciones resultantes también mostraban hipersensibilidad a fosfomicina, lo que llevó a ensayar la pareja tobramicina-fosfomicina como tratamiento tras la evolución secuencial, siendo más eficaz que la tobramicina en solitario.

En suma, este artículo caracteriza un *trade-off* fenotípico robusto (sensibilidad colateral a tobramicina), asociado a la adquisición de resistencia a ceftazidima en mutantes de *P. aeruginosa* resistentes a distintos antibióticos. Con él se realza la trascendencia que entraña la búsqueda de posibles coerciones a la evolución de la resistencia a antibióticos en bacterias, a fin de diseñar racionalmente tratamientos basados en convergencias fenotípicas.

Aportaciones específicas:

Trabajo experimental: Sanz-García, F. y Hernando-Amado, S. contribuyeron a la labor experimental. Concretamente, mi participación se centró en los estudios de la evolución secuencial en presencia de tobramicina-ceftazidima y en los análisis e interpretación de los resultados.

Elaboración del manuscrito: todos los autores contribuyeron a la escritura y corrección del manuscrito.

MICROBIOLOGY

Rapid and robust evolution of collateral sensitivity in *Pseudomonas aeruginosa* antibiotic-resistant mutants

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The analysis of trade-offs, as collateral sensitivity, associated with the acquisition of antibiotic resistance, is mainly based on the use of model strains. However, the possibility of exploiting these trade-offs for fighting already resistant isolates has not been addressed in depth, despite the fact that bacterial pathogens are frequently antibiotic-resistant, forming either homogeneous or heterogeneous populations. Using a set of *Pseudomonas aeruginosa*-resistant mutants, we found that ceftazidime selects pyomelanogenic tobramycin-hypersusceptible mutants presenting chromosomal deletions in the analyzed genetic backgrounds. Since pyomelanogenic resistant mutants frequently coexist with other morphotypes in patients with cystic fibrosis, we analyzed the exploitation of this trade-off to drive extinction of heterogeneous resistant populations by using tobramycin/ceftazidime alternation. Our work shows that this approach is feasible because phenotypic trade-offs associated with the use of ceftazidime are robust. The identification of conserved collateral sensitivity networks may guide the rational design of evolution-based antibiotic therapies in *P. aeruginosa* infections.

INTRODUCTION

Antibiotic effectiveness, currently compromised by the spread of antibiotic resistance (AR), requires not only innovation but also conservation, which may allow for improved use of current antibiotics (*1*). For this conservation, understanding of the trade-offs associated with AR acquisition—such as increased susceptibility to a second drug after use of the first, a phenomenon first described in the 1950s as collateral sensitivity (*2*)—might be particularly relevant. Various studies have been undertaken trying to exploit the evolutionary constraint imposed by collateral sensitivity patterns (*3*, *4*), such as combinatory therapy (*5*, *6*) or alternating collaterally susceptible drug pairs (*7*–*9*).

Despite progress in study of the collateral sensitivity phenomenon, some questions remain to be answered. In particular, there is still only limited information on the evolutionary conservation of collateral sensitivity patterns, not only between different species (*10*, *11*) but also within different members from the same species (*12*–*15*). In the case of *Pseudomonas aeruginosa*, some studies have revealed that isolates of this pathogen obtained from patients with chronic infections and treated with distinct antibiotic classes present convergence in their collateral sensitivity phenotypes (*9*), while others have described major differences in the collateral sensitivity patterns associated with the acquisition of resistance to one antibiotic between replicates of the same strain of *P. aeruginosa* evolving in parallel (*13*). These discrepancies may lie in the degree of reproducibility of the evolutionary pathways leading to AR. In this respect, it is known that the type of resistance mutations present in a given genetic background may be restricted because of epistatic interactions (*10*, *16*–*21*) and that the cumulative acquisition of AR mutations in different loci reduces the variety of pathways, leading to AR (*22*, *23*). Since contingency may be relevant not only for AR evolution but also for the acquired collateral sensitivity phenotype (*24*), knowing the degree of conservation of collateral sensitivity patterns associated with the use of a specific drug in different genetic backgrounds, particularly in the case of mutants already presenting a phenotype of AR, is of special interest.

In a previous study, we observed that *P. aeruginosa* PA14 populations experimentally evolved in the presence of ceftazidime displayed a robust collateral sensitivity to amikacin (*25*), as a consequence of selection of large chromosomal deletions upon 1 day of adaptive laboratory evolution (ALE) (*25*). The chromosomal deletions included *hmgA*, which encodes an enzyme whose lack of activity leads to the hyperproduction of the brown pigment pyomelanin (*26*); *galU*, whose inactivation reduces ceftazidime susceptibility (*27*); and *mexXY*, which encodes a multidrug resistance (MDR) efflux pump that contributes to *P. aeruginosa* intrinsic aminoglycosides resistance (*28*). Up to 13% of patients with cystic fibrosis (CF) are infected by *P. aeruginosa* pyomelanin-producing mutants (*29*) that, in agreement with the phenotype of the mutants selected after ALEs, are also hypersusceptible to aminoglycosides (*30*). Although the fact that pyomelanin increases resistance to oxidative stress and favors bacterial persistence in chronic lung infections (*26*) has been considered the most likely explanation for in vivo selection of those mutants, this genetic event has also been reported in different strains of *P. aeruginosa* subjected to ALE in the presence of other β-lactams (*31*–*34*). Therefore, it remains to be established whether these deletions are selected by the antibiotic treatment or merely represent an adaptation to the lung environment of patients with CF (*35*), as was previously proposed (*34*, *35*). Even further, it remains to be answered whether these deletions are selected upon ceftazidime treatment in different genetic backgrounds of *P. aeruginosa*, in particular, in mutants resistant to other antibiotics; or on the contrary, whether the evolutionary robustness of this genetic event, and of its phenotypic effects, is limited. In this study, by constructing a set of resistant mutants previously identified in different ALE experiments of *P. aeruginosa* PA14 (*16*, *36*) in the presence of antibiotics, and by submitting them, as well as the wild-type PA14 strain, to ALE in the presence of ceftazidime, we have determined the robustness of an early event of ceftazidime resistance evolution that is associated with collateral sensitivity to tobramycin. Further, by the recreation of heterogeneous pyomelanogenic populations belonging to each genetic background and the alternation of tobramycin with ceftazidime, we found that

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driving evolution toward hypersusceptibility to the first drug is generally feasible, at least in the genetic backgrounds analyzed. This finding supports the possibility of rationally designing treatments based on collateral sensitivity convergence in *P. aeruginosa*.

RESULTS

As mentioned above, the conservation of collateral sensitivity to a given drug within different genetic backgrounds of a species may be contingent on the degree of conservation of the evolutionary routes toward resistance to the first drug used for selection. We and others have reported the early selection of large chromosomal deletions that contain genes encoding the intrinsic aminoglycosides resistance efflux pump MexXY (*28*), when ALE experiments in the presence of different β-lactams, including ceftazidime (*31*), piperacillin (*32*), or meropenem (*33*, *34*), were performed. These data suggest that this genetic event could be one of the first evolutionary steps in the evolution of *P. aeruginosa* toward β-lactam resistance. However, since these studies were limited to a single wild-type genetic background, it remained to be analyzed whether a similar evolutionary pattern could be followed by *P. aeruginosa* PA14 strains presenting different genetic backgrounds, in particular, different antibiotic-resistant mutants. That being so, collateral sensitivity to aminoglycosides, such as tobramycin, a drug that forms part of usual therapy regimens against *P. aeruginosa* (*37*), would also be conserved.

Construction and characterization of *P. aeruginosa* mutants

Our first objective was to analyze the evolutionary conservation of ceftazidime resistance evolution in different genetic backgrounds, consisting of mutants derived from different *P. aeruginosa* PA14 ALEs in the presence of antibiotics (see table S1). It has been recently suggested that AR mutations may associate with either robust or variable collateral sensitivity patterns in different genetic backgrounds, depending on whether they lead to "target" or "regulatory alterations," respectively (*10*). Taking this hypothesis into account and resorting to previous different in-house ALE experiments (*16*, *36*), we constructed a broad spectrum of strains containing single mutations that affect regulatory proteins (NfxB, ParR, or MexZ), nonregulatory proteins (NuoD or OrfN), or simultaneously containing both types of mutations. The mutant containing mutations in *nfxB*, *phoQ*, *frr*, and *pmrB* was dubbed MDR6, and the mutant containing mutations in *fusA*, *orfN*, *pmrB*, *mexZ*, *gabP*, *ptsP*, and *nuoD* was dubbed MDR12 (see table S1 for detailed information). The susceptibility of each mutant to different antibiotics, including those of interest in this study—tobramycin and ceftazidime—is shown in Table 1.

Table 1. MICs (μg/ml) of different antibiotics for the single and multiple *P. aeruginosa* PA14 mutants used in this work. MICs ≥2-fold of the MICs for the wild-type PA14 strain are highlighted in bold. TOB, tobramycin; TGC, tigecycline; CAZ, ceftazidime; CIP, ciprofloxacin; IPM, imipenem.

	TOB	TGC	CAZ	CIP	IPM
PA14	1	6	1	0.094	0.75
nfxB177	1	**32**	1.5	**3**	1
parR87	1.5	8	1	0.125	**2**
orfN50	**3**	**32**	**3**	**0.19**	**2**
nuoD184	**2**	4	1	0.047	0.75
mexZ43	1.5	8	1	**0.38**	**1.5**
MDR6*	**2**	**48**	1.5	**0.19**	**1.5**
MDR12†	**32**	**64**	1	**0.5**	**1.5**

*MDR6 mutant presents mutations in *nfxB*, *phoQ*, *frr*, and *pmrB*.
†MDR12 mutant presents mutations in *fusA*, *orfN*, *pmrB*, *mexZ*, *gabP*, *ptsP*, and *nuoD*.

Evolutionary robustness of first steps of *P. aeruginosa* ceftazidime resistance evolution and collateral sensitivity to tobramycin

To determine whether chromosomal deletions containing *mexXY* would be early selected during *P. aeruginosa* evolution in presence of ceftazidime in the set of mutants mentioned above, as it was the case in the wild-type strain PA14 (*25*), four biological replicates of each single (*nfxB177*, *parR87*, *mexZ43*, *orfN50*, and *nuoD184*) and multiple mutants (MDR6 and MDR12), and the wild-type PA14 strain, were subjected to ALE in presence or absence of ceftazidime (a total of 64 populations) for 3 days. Upon 1 day of experimental evolution, almost every *P. aeruginosa* population challenged with antibiotic hyperproduced pyomelanin (27 of 32 populations; Fig. 1A). This result is consistent with the presence of deletions that include *hmgA*, as those described in our previous study, because pyomelanin accumulation is due to the lack of homogentisate 1,2-dioxygenase activity provided by HmgA (*26*). Since we had previously determined a cause-effect relationship between the presence of chromosomal deletions containing *hmgA* and *mexXY* and the hyperproduction of pyomelanin and hypersusceptibility to aminoglycosides, respectively (*25*), the susceptibility of each final population to tobramycin was analyzed. All the pyomelanogenic populations obtained after the short-term evolution in presence of ceftazidime were resistant to ceftazidime and hypersusceptible to tobramycin, when compared to the parental strain from which they evolved (Fig. 1B and table S2), even when the mutants were originally less susceptible to tobramycin than the wild-type *P. aeruginosa* PA14 strain. In particular, tobramycin minimal inhibitory concentration (MIC) was reduced by up to 4-fold in PA14, 2.6-fold in *nfxB177*, 3-fold in *parR87*, 7.9-fold in *orfN50*, 6-fold in *mexZ43*, 5.3-fold in MDR6, and 10.7-fold in MDR12. Consistent with the linkage between pyomelanin production and deletion of a chromosomal region containing *mexXY*, nonpyomelanogenic populations (*parR87*, replicate 1; and *nuoD184*, all replicates) were not tobramycin hypersusceptible (Fig. 1B and table S2). Further analysis of the results from the experimental evolution study has revealed that six of eight genetic backgrounds presented a significantly ($P < 0.01$ in all cases) reduced MIC to tobramycin, compared to their parental strains, after the ceftazidime short-term evolution. To further analyze whether chromosomal deletions containing *mexXY* could be associated with the phenotype of hypersusceptibility, every evolved population, as well as their original parental strain, was genotyped (fig. S1A). A 163-bp polymerase chain reaction (PCR) fragment corresponding to *mexXY* was detected in every parental strain, as well as in nonpyomelanogenic populations (*parR87*, replicate 1; and *nuoD184*, all replicates) and in a pyomelanogenic mixed population (PA14, replicate 2). Consistent with the observed increase in tobramycin susceptibility of most evolved populations (table S2), every pyomelanogenic tobramycin-hypersusceptible population lacked *mexXY* (fig. S1A). Overall, these results suggest that chromosomal deletions containing *mexXY* are consistently selected at first steps

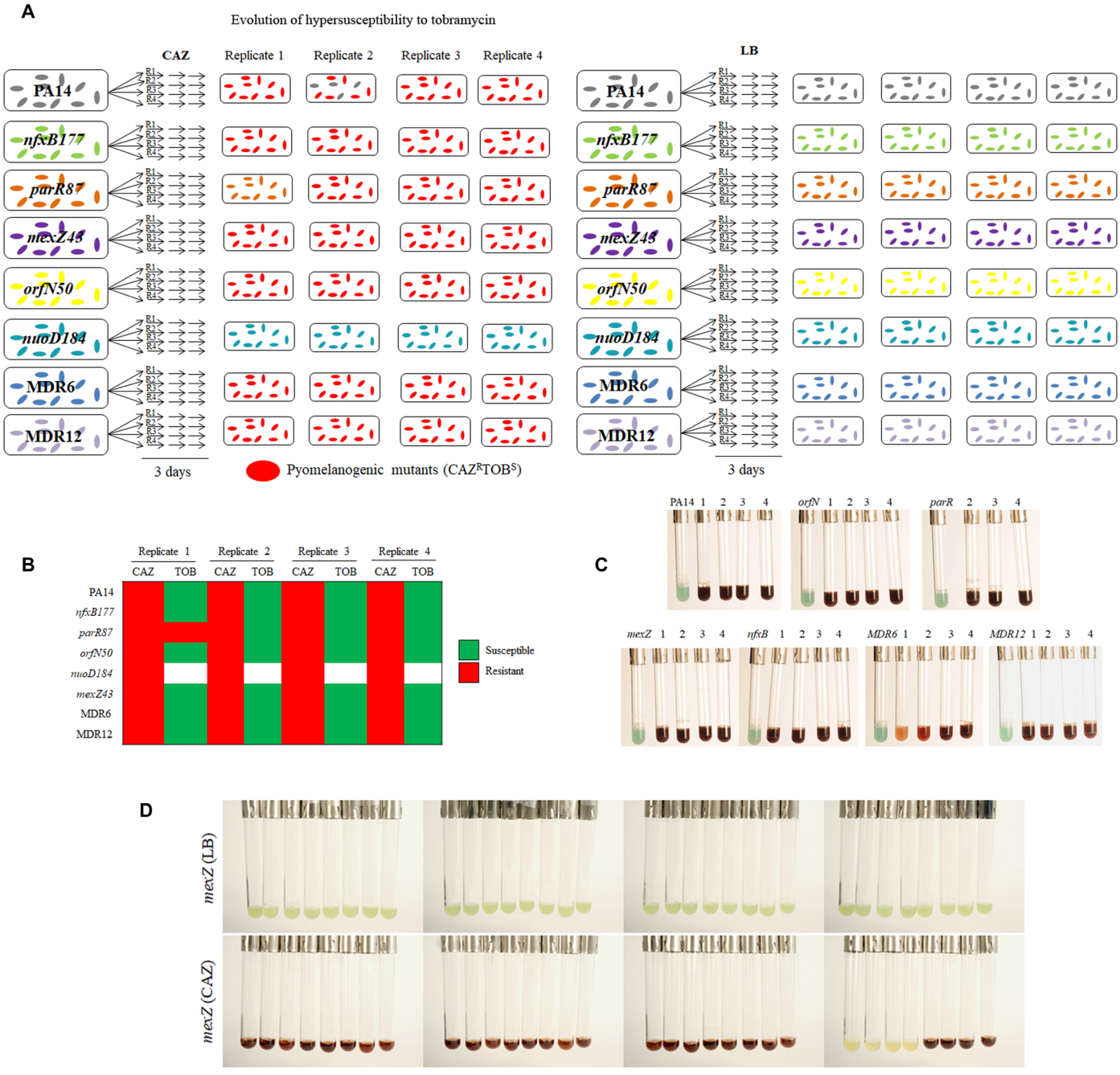

Fig. 1. Analysis of early steps in the evolution of *P. aeruginosa* wild-type strain and antibiotic-resistant mutants in the presence of CAZ. (**A**) Scheme of the resultant phenotype after the evolution of PA14, single (*nfxB177*, *parR87*, *mexZ43*, *orfN50*, and *nuoD184*) and multiple (MDR6 and MDR12) mutants, in the presence of CAZ [left part of (A)] or in the absence of antibiotic [LB; right part of (A)] for 3 days (see Materials and Methods). Pyomelanin hyperproduction was observed in 27 of 32 populations evolved in the presence of CAZ (red-colored cells). (**B**) Diagram showing convergence toward hypersusceptibility to TOB in the different genetic backgrounds and replicates, analyzed after short-term evolution on CAZ. In all cases, acquisition of a pyomelanogenic phenotype is associated with collateral sensitivity to TOB, irrespective of the genetic background of the evolving strain. MIC values of TOB and CAZ are included in table S2. (**C**) Isolation of pyomelanogenic clones from each 27 pyomelanogenic population [left part of (A); red-colored cells] obtained in the presence of CAZ. As shown in the figure, the early steps of evolution in presence of CAZ of *P. aeruginosa*, which lead to collateral sensitivity to TOB and pyomelanin production, are conserved among the different antibiotic-resistant mutants analyzed. (**D**) Pyomelanogenic phenotype of *mexZ43* mutant after 1-day evolution in the presence of CAZ or in the absence of antibiotic (LB). Pyomelanin hyperproduction was observed in 28 of 32 populations evolved on CAZ. Photo credits for (C) and (D): Inés Poveda, Centro Nacional de Biotecnología. Permission for using these images is not required.

of ceftazidime resistance evolution in *P. aeruginosa*, at least in the genetic backgrounds analyzed. To further test whether this observation could be conditioned by the number of replicates used during the assay for each genetic background, 32 replicates of one of the mutants (*mexZ43*) were subjected to evolution under the same conditions used before, in presence or absence of ceftazidime. We observed that upon 1 day of experimental evolution, a high number of *mexZ43* populations challenged with antibiotic hyperproduced pyomelanin (28 of 32 populations; Fig. 1D), just 4 of 32 populations did not become pyomelanogenic (Fig. 1D). Although we are aware that there is a space for unpredictability of ceftazidime resistance evolution in the mutants analyzed, the results here described suggest that alternative genetic evolutionary trajectories in ceftazidime resistance evolution may be limited, and that phenotypic convergence toward collateral sensitivity to tobramycin is robust in *P. aeruginosa*, even in the case of antibiotic-resistant mutants, as those here analyzed (table S1), after ceftazidime treatment (table S2). However, we are fully aware that all mutants described here derive from *P. aeruginosa* PA14, and the generalization of our results to other strains will require the analysis of the effect of short-term ceftazidime evolution on a broad and diverse set of clinical strains of *P. aeruginosa*.

Evolution of heterogeneous pyomelanogenic populations of *P. aeruginosa* subjected to tobramycin/ceftazidime sequential evolution

We have shown that ceftazidime selects, in the short term, pyomelanogenic mutants presenting collateral sensitivity to tobramycin in *P. aeruginosa*, at least in the set of resistant mutants analyzed here (table S1). Nevertheless, there is an extensive heterogeneity within populations of *P. aeruginosa* in the CF lungs (*38*), frequently including mutants already presenting a pyomelanogenic phenotype (*29*, *30*), which is usually associated with a reduced susceptibility to ceftazidime. Since this heterogeneity might impair the chances of exploiting the observed tobramycin collateral sensitivity associated with the use of ceftazidime in these heterogeneous populations, we tested the possibility of alternating the antibiotics, first, by using tobramycin and then second, ceftazidime, for reducing the chances of pyomelanogenic heterogeneous populations to escape from the antibiotic challenge.

The strategy would consist of three stages: A first step on tobramycin may force extinction of the ceftazidime-resistant (tobramycin-hypersusceptible) part of the populations, a second step on ceftazidime may drive evolution toward tobramycin hypersusceptibility, and then the extinction of the tobramycin-hypersusceptible cells after switching back to tobramycin (Fig. 2) would be expected. Although this strategy would be also potentially applicable to populations not resistant to ceftazidime, being reduced to a first step on ceftazidime followed by a second one on tobramycin, we decided to analyze its effectiveness in a more complex situation of clinical relevance, as it is the case of heterogeneous pyomelanogenic populations. In particular, diverse populations derived from the set of mutants were analyzed in this work (see table S1). To that end, we isolated a pyomelanogenic clone from each of the 27 pyomelanogenic populations obtained after ceftazidime short-term evolution (Fig. 1A). To note here that although the original clones from which these populations were derived belonged to a diverse set of genetic backgrounds, consisting of antibiotic-resistant mutants that contain mutations in regulatory, nonregulatory, or both types of proteins (Table 1 and table S1), all the isolated clones that hyperproduced pyomelanin

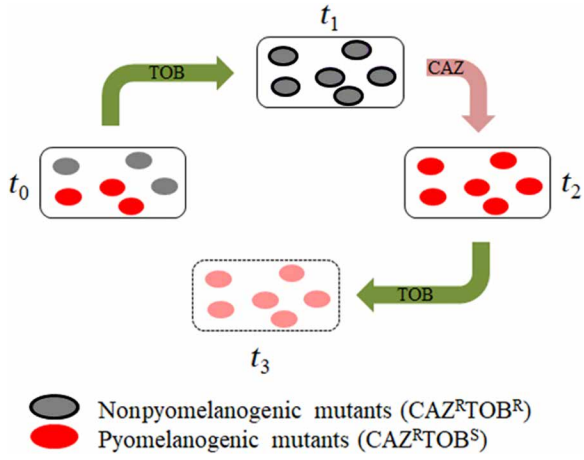

Fig. 2. General model illustrating evolution of heterogeneous pyomelanogenic populations of *P. aeruginosa* subjected to TOB/CAZ sequential evolution. Evolution of a heterogeneous population containing a pyomelanogenic (CAZ resistant) subpopulation starts when TOB is added at time zero (t_0). In the presence of TOB, there is an extinction of TOB-hypersusceptible pyomelanogenic mutants (red-colored cells) and TOB becomes ineffective (t_1). Then, treatment is switched to CAZ, and TOB-resistant cells (contoured gray-colored cells) become TOB-hypersusceptible (t_2). Treatment would be switched back to TOB, resulting in the elimination of TOB-hypersusceptible cells (t_3). This strategy would also be potentially applicable to initial populations not resistant to CAZ (t_1), being reduced to a first step on CAZ (leading to t_2), followed by a second step on TOB (resulting in t_3).

(Fig. 1C) were significantly ($P < 0.001$ in all cases) more susceptible to tobramycin than their respective parental strain (table S3), and lacked *mexXY* (fig. S1B). Then, we recreated a total of 27 heterogeneous pyomelanogenic populations by mixing each of the 27 pyomelanogenic clones with its respective parental strain in a 1:1 ratio. The heterogeneous populations were dubbed PA14 +1 to +4, *nfxB177* +1 to +4, *parR87* +2 to +4, *mexZ43* +1 to +4, *orfN50* +1 to +4, MDR6 +1 to +4, and MDR12 +1 to +4. These populations were first subjected to tobramycin short-term evolution for 3 days (see Materials and Methods), and the capacity of these populations to either evolve toward tobramycin resistance or to go extinct was analyzed (Fig. 3). Since pyomelanogenic clones are less susceptible to ceftazidime (i.e., >256 μg/ml in all the *parR87* clones; see "b" data in table S4) than the parental strain from which they evolved (1 μg/ml in *parR87*; see "a" data in table S4), ceftazidime MIC values were used to verify the extinction of the pyomelanogenic part of each population. After 3 days of evolution in the presence of tobramycin, the ceftazidime MICs for every population (i.e., 1 μg/ml in *parR87* heterogeneous populations; see the "First step (TOB)" data in table S4) were close to the ceftazidime MIC for the parental strain [compare First Step (TOB) to the "a and b" MIC data in table S4]. These data support that the pyomelanogenic part of every population is extinct after the first step of sequential evolution. To further confirm the extinction of the pyomelanogenic clones, the phenotype (green/yellow versus brown color) of 20 clones from each of the 27 populations, a total of 540 clones, were isolated and grown in liquid medium (brown color is poorly appreciated in colonies) to detect any escape of ceftazidime-resistant cells from tobramycin treatment. As shown in Fig. 3B, none of the clones produced pyomelanin, confirming the extinction of the pyomelanogenic part of every population, a fact that was previously hinted by the ceftazidime MIC values of the resultant populations

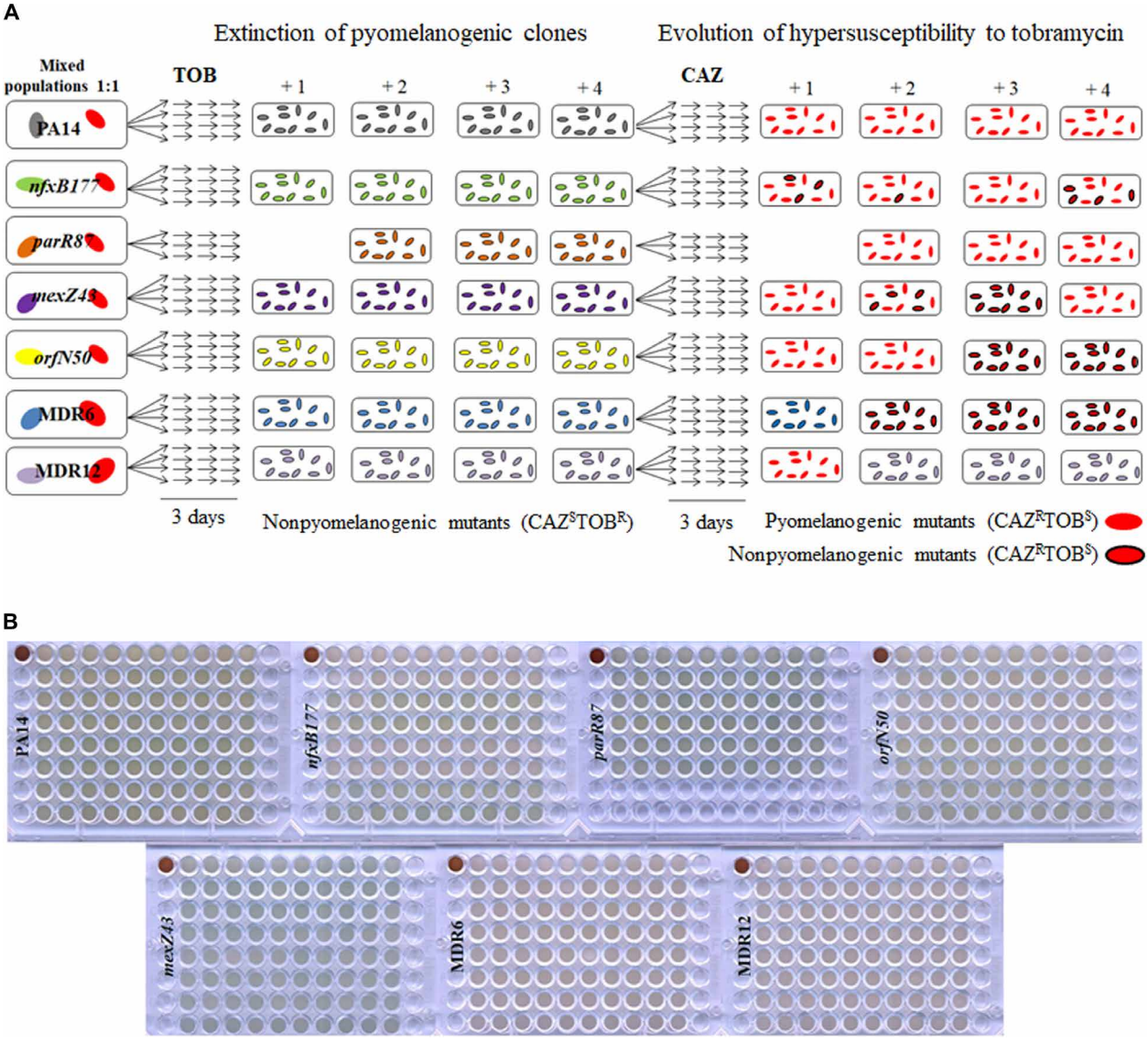

Fig. 3. Analysis of TOB/CAZ sequential evolution of heterogeneous pyomelanogenic populations of *P. aeruginosa*. (**A**) Diagram showing the evolution of heterogeneous populations (dubbed +1, +2, +3, and +4) containing each parental strain: PA14, *nfxB177*, *parR87*, *mexZ43*, *orfN50*, MDR6, or MDR12 and four individual pyomelanogenic clones belonging to the same genetic background, during first step of sequential evolution in the presence of TOB (left) and second step in the presence of CAZ (right), for 6 days (see Materials and Methods). In the case of *parR87*, only three pyomelanogenic clones from independent CAZ-evolved populations (see Fig. 1, A and C) could be isolated. Hypersusceptibility to TOB (contoured and noncontoured red-colored cells) is observed in 23 of 27 populations (see table S4), and pyomelanin production (noncontoured red-colored cells) is observed in 17 of 27 populations. (**B**) Analysis of extinction of the pyomelanogenic part of the heterogeneous pyomelanogenic populations after a first step of sequential evolution in the presence of TOB [(A) section, left]. The phenotype (color) of 20 clones isolated from each heterogeneous population, after 3 days of TOB evolution, was observed in liquid medium and compared with the color (brown) of each pyomelanogenic parental strain (upper left corner of each plate). In agreement with data shown in table S4, which points to the extinction of pyomelanogenic populations by comparison of CAZ MIC value of each heterogeneous population with the ones of their parental strains and pyomelanogenic clones, the color of the 540 clones analyzed indicated that pyomelanogenic clones were extinct after first step of sequential evolution on TOB. Photo credits for (B): Fernando Sanz-García, Centro Nacional de Biotecnología.

(see table S4). Besides that, each resultant population presented an increased tobramycin MIC, up to 48-fold, depending on the genetic background and replicate (table S4).

At this point, we specifically focused on the switch from tobramycin to ceftazidime, the second step of the sequential evolution (Fig. 2). Although we had observed conservation of tobramycin collateral sensitivity after evolution in presence of ceftazidime within the analyzed set of mutants of *P. aeruginosa* (Fig. 1 and table S2), a critical point would be to determine whether this genetic event would also be selected by ceftazidime in the tobramycin-resistant mutants obtained after the first step of evolution of the populations in the presence of tobramycin. Hence, we switched the selective pressure from tobramycin to ceftazidime (see Materials and Methods). As shown in Fig. 3, 17 of 27 populations hyperproduced pyomelanin. A second critical point emerged here: The degree of sensitization to tobramycin of highly resistant mutants to the said antibiotic was

uncertain. To determine whether these populations presented an increased susceptibility to tobramycin, as it was observed in the wild-type PA14 background and in most of mutants analyzed in this work (Fig. 1 and table S2), the tobramycin MIC was determined in all the evolved populations. Twenty-three of 27 populations presented an important increase in their sensitivity to tobramycin after switching the selective pressure from tobramycin to ceftazidime, reducing the MIC by up to 128-fold in PA14, 48-fold in *nfxB177*, 21-fold in *parR87*, 96-fold in *orfN50*, 43-fold in *mexZ43*, 4-fold in MDR6, and 11-fold in MDR12 (Fig. 4 and table S4). This analysis revealed that five of seven heterogeneous pyomelanogenic populations presented a substantially reduced MIC to tobramycin after the second step of sequential evolution on ceftazidime (table S4). These results suggest that it could be possible to exploit the tobramycin collateral sensitivity associated with the use of ceftazidime by switching back selective pressure to tobramycin, although there may be some limitations depending on the genetic background. This was the case of the multiple resistant mutants MDR6 and MDR12, which did not present a relevant reduction in tobramycin MIC after the switch to ceftazidime.

The fact that some populations were hypersusceptible to tobramycin, even without having suffered chromosomal deletions containing *mexXY* (6 of 27 populations; Fig. 3 and fig. S1C) or being mixed populations (4 of 27 populations; Fig. 3 and fig. S1C), indicates that reciprocal collateral sensitivity between ceftazidime and tobramycin may occur even in the absence of these deletions, a feature that remains to be explored in detail. Overall, our results indicate that this strategy could potentially be applicable from complex situations similar to the ones explored in the current work (heterogeneous pyomelanogenic ceftazidime-resistant populations) to other ones (populations not resistant to ceftazidime).

Cross-resistance and collateral sensitivity patterns of *P. aeruginosa* after tobramycin/ceftazidime sequential evolution

We have recently described that *P. aeruginosa* replicate populations subjected to ribosome-targeting antibiotics may present common changes in the susceptibility to other antibiotics, besides those used along selection (*16*, *36*). However, important differences have been described in the collateral sensitivity phenotype among replicate populations of the same *P. aeruginosa* strain adapted to one antibiotic (*13*). We have recently reported that a loss-of-function mutant of *P. aeruginosa* PA14, differing from its parental strain in the activity of just one regulator, not directly linked to AR, presents different patterns of collateral sensitivity and cross-resistance phenotype when it acquires resistance to ribosome-targeting antibiotics (*16*). Since historical contingency may restrict the evolution of collateral sensitivity and cross-resistance phenotypic outcomes, we wondered whether the populations obtained after tobramycin and ceftazidime sequential evolution, besides presenting a convergent hypersusceptibility to tobramycin, may converge toward the phenotypes of cross-resistance or collateral sensitivity to antibiotics found in other structural families. To address this question, the MICs of a set of antibiotics were determined for the 27 populations. A general pattern of cross-resistance to aztreonam, imipenem, and chloramphenicol, as well as significant collateral sensitivity to fosfomycin, tobramycin, and tetracycline, was observed ($P < 0.0001$ in all cases) (Fig. 5 and table S5). Since the combination fosfomycin-tobramycin has been found to be synergistic against biofilms of CF *P. aeruginosa* strains (*39*) and against *P. aeruginosa* PAO1 in anaerobic environments (*40*), we propose that the switch back to tobramycin (Fig. 2) could also be replaced, if necessary, by the combination fosfomycin-tobramycin. To analyze the relative efficacy of the two possible options, we

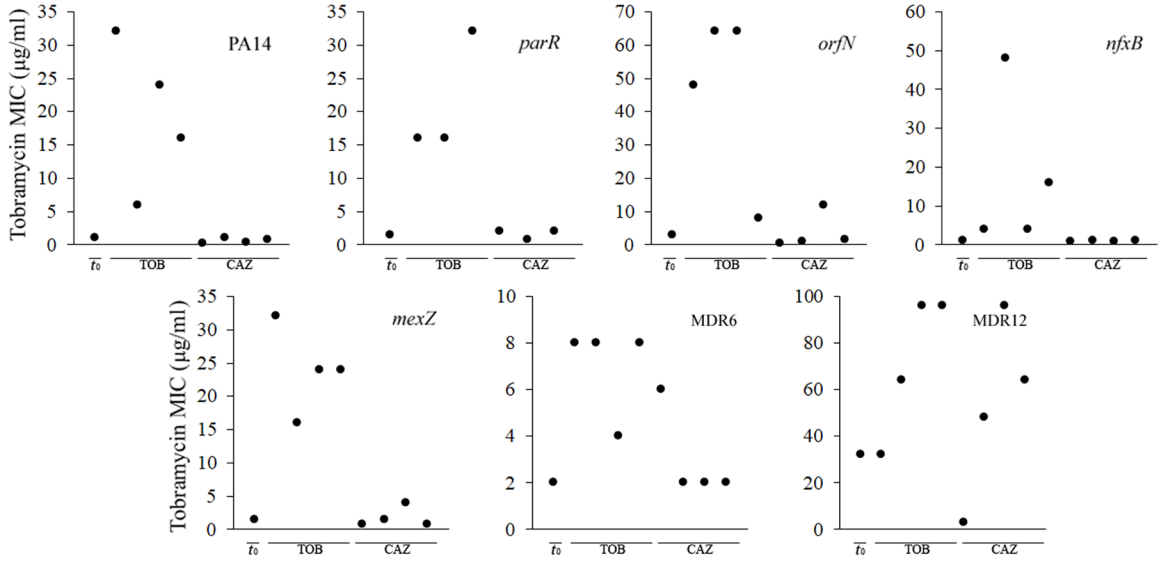

Fig. 4. MICs (μg/ml) of TOB for heterogeneous pyomelanogenic populations of *P. aeruginosa* after TOB/CAZ sequential evolution. Evolution of TOB MICs (μg/ml) of heterogeneous pyomelanogenic populations after sequential evolution on TOB/CAZ (see Fig. 3). Each plot shows the TOB MIC values for a parental strain (PA14, *parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, or MDR12), indicated as t_0 in the *x* axis, and for four heterogeneous pyomelanogenic populations (represented as black circles) after first evolution on TOB (indicated as TOB in the *x* axis), followed by second evolution on CAZ (indicated as CAZ in the *x* axis). Only three heterogeneous pyomelanogenic populations of *parR87* were analyzed. TOB MICs decreased after switching from TOB to CAZ by up to 128-fold in PA14, 48-fold in *nfxB177*, 21-fold in *parR87*, 96-fold in *orfN50*, 43-fold in *mexZ43*, 4-fold in MDR6, and 11-fold in MDR12. MIC values are shown in table S4.

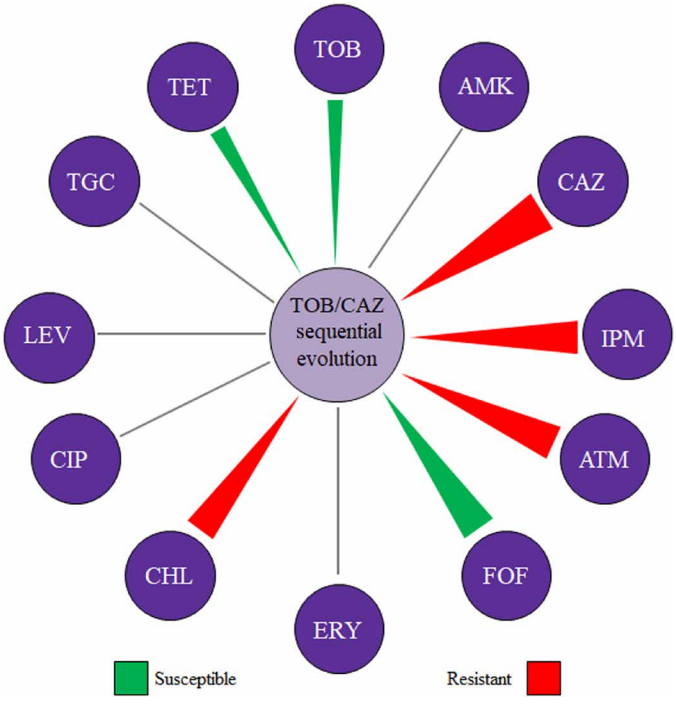

Fig. 5. Diagram showing the degree of convergence of cross-resistance and collateral sensitivity in heterogeneous pyomelanogenic populations of *P. aeruginosa* obtained after TOB/CAZ sequential evolution. Collateral sensitivity and cross-resistance to antibiotics from different structural families were analyzed in the 27 populations obtained after sequential evolution. A population is classified as "susceptible" or "resistant" when there was an MIC change with respect to the parental strain value. Triangles indicate antibiotics where a predominant change toward resistance (red) or susceptibility (green) with respect to the parental strain was observed. Thickness of the triangle depends on the percentage of conservation of said phenotype. MIC values (μg/ml) are included in table S5. AMK, amikacin; ATM, aztreonam; FOF, fosfomycin; ERY, erythromycin; CHL, chloramphenicol; LEV, levofloxacin; TET, tetracycline.

subjected the resultant populations obtained after tobramycin/ceftazidime sequential evolution to ALE in either tobramycin or the combination fosfomycin-tobramycin, applying twice the MIC of each parental strain. We observed that 8 of 27 populations were able to escape from the switch back to tobramycin (*orfN50* +3, *mexZ43* +2 and +3, MDR6 +1 and +3, and MDR12 +2 to +4). This result agrees with the fact that although most of the populations (23 of 27) presented an important reduction in the tobramycin MIC after sequential evolution on tobramycin and ceftazidime, by up to 128-fold in PA14, 48-fold in *nfxB177*, 21-fold in *parR87*, 96-fold in *orfN50*, 43-fold in *mexZ43*, 4-fold in MDR6, and 11-fold in MDR12 (Fig. 4), tobramycin MIC values were close to those of parental strains. None of the populations survived the combination fosfomycin-tobramycin, possibly due to the synergistic effect of these antibiotics (*39*). We therefore propose that use of the fosfomycin-tobramycin combination is more effective than switching back to tobramycin after that treatment.

DISCUSSION

Bacterial evolution is known to be one of the main causes of the current AR problem; but in-depth analysis of this evolution could also help to tackle this issue through the exploitation of the evolutionary trade-offs (as collateral sensitivity) associated with AR acquisition (*2*, *4*). However, the feasibility of this approach requires the collateral sensitivity phenotypes of different resistant mutants to be robust and reproducible (*41*). In this study, we describe the robustness of collateral sensitivity to tobramycin associated with the short-term use of ceftazidime in an array of *P. aeruginosa* antibiotic-resistant mutants, chosen on the basis of their differences both in resistance phenotype and in the functions affected by the mutations that they harbor. We propose that the observed evolutionary trade-offs could be exploited for treating both clonal and heterogeneous pyomelanogenic infections. Patients with CF are usually infected by heterogeneous *P. aeruginosa* populations (*38*) that include pyomelanogenic mutants (*29*, *30*), which frequently present resistance to β-lactams, a feature that could compromise the use of ceftazidime. However, we have found that it is possible to drive the extinction of the pyomelanogenic mutants, first, by using tobramycin and then second, by driving the evolution of the remaining population toward tobramycin hypersusceptibility, using ceftazidime. A bottleneck for the application of this strategy would be the durability over time of tobramycin hypersusceptibility. However, it is important to highlight that the populations obtained after tobramycin/ceftazidime alternation present also collateral sensitivity to fosfomycin. We observed that it is possible to replace the switch back to tobramycin by a fosfomycin-tobramycin combination, which results in higher efficacy. These results point to the possibility of exploiting specific evolutionary trade-offs for tackling the problem of AR. However, we are aware that a detailed analysis determining the degree of conservation of the short-term ceftazidime resistance evolution in a broad and diverse set of clinical strains of *P. aeruginosa* would be required. Overall, our results and those of others, previously described (*41*) suggest that the analysis of phenotypic convergence and, in particular, the aspects that deal with the collateral sensitivity of *P. aeruginosa* AR mutants, is an important step forward in the rational design of therapeutic approaches capable of reducing the AR burden.

MATERIALS AND METHODS

Growth conditions and antibiotic susceptibility assays

Bacteria were grown in LB at 37°C, with shaking at 250 rpm in glass tubes. MICs of ceftazidime, aztreonam, imipenem, tobramycin, amikacin, tigecycline, tetracycline, ciprofloxacin, levofloxacin, chloramphenicol, fosfomycin, and erythromycin were determined at 37°C in Mueller-Hinton (MH) agar, using E-test strips (MIC Test Strip, Liofilchem).

Mutant construction

Four single mutants of *P. aeruginosa* (*nfxB177*, *parR87*, *mexZ43*, and *nuoD184*) were constructed by inserting each mutant allele (table S1) by homologous recombination into the wild-type PA14, while the *orfN50* single mutant was previously obtained (*16*). Mutant alleles were obtained by PCR from previous in-house evolved populations (*16*, *36*), leaving approximately 500 bp upstream and downstream the corresponding single-nucleotide polymorphism, using the oligonucleotides described in table S6. PCR products containing Hind III restriction sites were cloned into the Hind III–digested and dephosphorylated pEX18Ap vector (*42*) and then introduced by transformation into the conjugative *Escherichia coli* S17-1 strain. Subsequently, conjugation and mutant selection were performed, as described

elsewhere (*42*), using carbenicillin (350 μg/ml) and 10% sucrose. In all cases, the presence of the mutations was confirmed by Sanger sequencing. To obtain mutants containing multiple mutations, 10 independent resistant clones from end point evolved populations (*16*) on tobramycin or tigecycline were selected and mutations confirmed by Sanger sequencing. From them, two multiple mutants (table S1), tobramycin or tigecycline resistant, respectively, were chosen. The mutant allele of *lasR* was replaced by homologous recombination with that of the wild type in the two selected clones, using the above-described strategy and oligonucleotides encompassed in table S6.

Short-term ALE in presence of ceftazidime

Five single mutants, two multiple mutants, and PA14, four replicates of each, were subjected to short-term ALE in presence or absence of ceftazidime, resulting in a total of 64 independent bacterial populations (32 populations grown in presence of ceftazidime and 32 control populations grown without antibiotic). Cultures were grown at 37°C and 250 rpm for 3 days. Every day, the cultures were diluted (1/125), adding 8 μl of bacteria in 1 ml of fresh LB, either containing or lacking ceftazidime at the concentration that hinders the growth of each *P. aeruginosa* genetic background under these culture conditions (4 μg/ml for PA14, *mexZ43*, and MDR12; 5 μg/ml for *nfxB177*, *orfN50*, and MDR6; 3 μg/ml for *parR87*; and 2 μg/ml for *nuoD184*). During the 3 days, the concentration of ceftazidime was maintained. Every replicate population was preserved at −80°C at the end of the experimental evolution. In addition, the MIC of the antibiotic used for selection in populations (ceftazidime) and of the one to which ceftazidime evolution gives rise to collateral sensitivity (tobramycin), was determined at 37°C in MH agar using E-test strips.

Sequential tobramycin/ceftazidime experimental evolution

Pyomelanogenic clones were isolated from every individual pyomelanogenic replicate population of each genetic background previously submitted to short-term evolution in the presence of ceftazidime, resulting in a total of 27 pyomelanogenic clones (see above). Overnight bacterial cultures from each pyomelanogenic clone and its parental strain were normalized to an optical density at 600 nm of 4.0 and then mixed in a 1:1 (pyomelanogenic clone:parental strain) ratio, obtaining 27 heterogeneous populations. Cultures were grown at 37°C and 250 rpm for 6 days. Every day, during the first 3 days, the cultures were diluted (1/125) in fresh LB containing the tobramycin concentration that hinders the growth of each *P. aeruginosa* genetic background under these culture conditions (1 μg/ml for PA14; 1.5 μg/ml for *nfxB177*, *parR87*, *mexZ43*, and MDR6; 4 μg/ml for *orfN50*; and 12 μg/ml for MDR12). During the 3 days, the concentration of tobramycin was maintained. At the end of the first step of sequential experimental evolution, every replicate population was preserved at −80°C, and the MIC of ceftazidime and tobramycin was determined at 37°C in MH agar using E-test strips. The 27 populations were grown, from glycerol stocks, and every day, during the last 3 days, the cultures were diluted (1/125) in fresh LB containing ceftazidime, as described in the above-mentioned section of Material and Methods (see the "Short-term ALE in presence of ceftazidime" section). Every final population was preserved at −80°C at the end of the second step of sequential experimental evolution, and the MIC of tobramycin was determined at 37°C in MH agar using E-test strips.

Analysis of the presence/absence of *mexXY* in the evolved populations

The presence of chromosomal deletions including *mexXY* in the different genetic backgrounds and their respective evolved populations was analyzed by determining the absence of a 163-bp PCR fragment belonging to *mexXY* in 2% agarose gel. Primers used for *mexXY* genotyping are included in table S6.

Statistical analysis

Data were subjected to pre hoc and post hoc analyses to identify relevant differences, using either analysis of variance (ANOVA), Friedman's, or χ^2 tests and Dunnett's or Fisher's exact test with Hochberg correction, as implemented in R.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/32/eaba5493/DC1

View/request a protocol for this paper from *Bio-protocol*.

REFERENCES AND NOTES

1. R. Laxminarayan, Antibiotic effectiveness: Balancing conservation against innovation. *Science* **345**, 1299–1301 (2014).
2. W. Szybalski, V. Bryson, Genetic studies on microbial cross resistance to toxic agents. I. Cross resistance of *Escherichia coli* to fifteen antibiotics. *J. Bacteriol.* **64**, 489–499 (1952).
3. M. Baym, L. K. Stone, R. Kishony, Multidrug evolutionary strategies to reverse antibiotic resistance. *Science* **351**, aad3292 (2015).
4. C. Pál, B. Papp, V. Lázár, Collateral sensitivity of antibiotic-resistant microbes. *Trends Microbiol.* **23**, 401–407 (2015).
5. C. Munck, H. K. Gumpert, A. I. N. Wallin, H. H. Wang, M. O. A. Sommer, Prediction of resistance development against drug combinations by collateral responses to component drugs. *Sci. Transl. Med.* **6**, 262ra156 (2014).
6. C. Barbosa, R. Beardmore, H. Schulenburg, G. Jansen, Antibiotic combination efficacy (ACE) networks for a *Pseudomonas aeruginosa* model. *PLOS Biol.* **16**, e2004356 (2018).
7. L. Imamovic, M. O. Sommer, Use of collateral sensitivity networks to design drug cycling protocols that avoid resistance development. *Sci. Transl. Med.* **5**, 204ra132 (2013).
8. S. Kim, T. D. Lieberman, R. Kishony, Alternating antibiotic treatments constrain evolutionary paths to multidrug resistance. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 14494–14499 (2014).
9. L. Imamovic, M. M. H. Ellabaan, A. M. Dantas Machado, L. Citterio, T. Wulff, S. Molin, H. Krogh Johansen, M. O. A. Sommer, Drug-driven phenotypic convergence supports rational treatment strategies of chronic infections. *Cell* **172**, 121–134.e14 (2018).
10. M. Knopp, D. I. Andersson, Predictable phenotypes of antibiotic resistance mutations. *mBio* **9**, e00770-18 (2018).
11. G. Apjok, G. Boross, Á. Nyerges, G. Fekete, V. Lázár, B. Papp, C. Pál, B. Csörgő, Limited evolutionary conservation of the phenotypic effects of antibiotic resistance mutations. *Mol. Biol. Evol.* **36**, 1601–1611 (2019).
12. N. L. Podnecky, E. G. A. Fredheim, J. Kloos, V. Sørum, R. Primicerio, A. P. Roberts, D. E. Rozen, Ø. Samuelsen, P. J. Johnsen, Conserved collateral antibiotic susceptibility networks in diverse clinical strains of *Escherichia coli*. *Nat. Commun.* **9**, 3673 (2018).
13. C. Barbosa, V. Trebosc, C. Kemmer, P. Rosenstiel, R. Beardmore, H. Schulenburg, G. Jansen, Alternative evolutionary paths to bacterial antibiotic resistance cause distinct collateral effects. *Mol. Biol. Evol.* **34**, 2229–2244 (2017).
14. V. Lázár, G. Pal Singh, R. Spohn, I. Nagy, B. Horváth, M. Hrtyan, R. Busa-Fekete, B. Bogos, O. Méhi, B. Csörgő, G. Pósfai, G. Fekete, B. Szappanos, B. Kégl, B. Papp, C. Pál, Bacterial evolution of antibiotic hypersensitivity. *Mol. Syst. Biol.* **9**, 700 (2013).
15. V. Lázár, I. Nagy, R. Spohn, B. Csörgő, A. Györkei, Á. Nyerges, B. Horváth, A. Vörös, R. Busa-Fekete, M. Hrtyan, B. Bogos, O. Méhi, G. Fekete, B. Szappanos, B. Kégl, B. Papp, C. Pál, Genome-wide analysis captures the determinants of the antibiotic cross-resistance interaction network. *Nat. Commun.* **5**, 4352 (2014).
16. S. Hernando-Amado, F. Sanz-García, J. L. Martínez, Antibiotic resistance evolution is contingent on the quorum-sensing response in *Pseudomonas aeruginosa*. *Mol. Biol. Evol.* **36**, 2238–2251 (2019).
17. T. Vogwill, M. Kojadinovic, R. C. MacLean, Epistasis between antibiotic resistance mutations and genetic background shape the fitness effect of resistance across species of *Pseudomonas*. *Proc. Biol. Sci.* **283**, 20160151 (2016).
18. S. Trindade, A. Sousa, K. B. Xavier, F. Dionisio, M. G. Ferreira, I. Gordo, Positive epistasis drives the acquisition of multidrug resistance. *PLOS Genet.* **5**, e1000578 (2009).

19. H. Ward, G. G. Perron, R. C. Maclean, The cost of multiple drug resistance in *Pseudomonas aeruginosa*. *J. Evol. Biol.* **22**, 997–1003 (2009).
20. M. L. Salverda, E. Dellus, F. A. Gorter, A. J. Debets, J. van der Oost, R. F. Hoekstra, D. S. Tawfik, J. A. de Visser, Initial mutations direct alternative pathways of protein evolution. *PLOS Genet.* **7**, e1001321 (2011).
21. S. Kryazhimskiy, D. P. Rice, E. R. Jerison, M. M. Desai, Microbial evolution. Global epistasis makes adaptation predictable despite sequence-level stochasticity. *Science* **344**, 1519–1522 (2014).
22. N. Jochumsen, R. L. Marvig, S. Damkiær, R. L. Jensen, W. Paulander, S. Molin, L. Jelsbak, A. Folkesson, The evolution of antimicrobial peptide resistance in *Pseudomonas aeruginosa* is shaped by strong epistatic interactions. *Nat. Commun.* **7**, 13002 (2016).
23. A. Novais, I. Comas, F. Baquero, R. Cantón, T. M. Coque, A. Moya, F. González-Candelas, J.-C. Galán, Evolutionary trajectories of beta-lactamase CTX-M-1 cluster enzymes: Predicting antibiotic resistance. *PLOS Pathog.* **6**, e1000735 (2010).
24. D. Nichol, J. Rutter, C. Bryant, A. M. Hujer, S. Lek, M. D. Adams, P. Jeavons, A. R. A. Anderson, R. A. Bonomo, J. G. Scott, Antibiotic collateral sensitivity is contingent on the repeatability of evolution. *Nat. Commun.* **10**, 334 (2019).
25. F. Sanz-García, S. Hernando-Amado, J. L. Martínez, Mutation-driven evolution of *Pseudomonas aeruginosa* in the presence of either ceftazidime or ceftazidime-avibactam. *Antimicrob. Agents Chemother.* **62**, e01379-18 (2018).
26. A. Rodríguez-Rojas, A. Mena, S. Martín, N. Borrell, A. Oliver, J. Blázquez, Inactivation of the *hmgA* gene of *Pseudomonas aeruginosa* leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection. *Microbiology* **155**, 1050–1057 (2009).
27. C. Alvarez-Ortega, I. Wiegand, J. Olivares, R. E. Hancock, J. L. Martínez, Genetic determinants involved in the susceptibility of *Pseudomonas aeruginosa* to β-lactam antibiotics. *Antimicrob. Agents Chemother.* **54**, 4159–4167 (2010).
28. N. Masuda, E. Sakagawa, S. Ohya, N. Gotoh, H. Tsujimoto, T. Nishino, Contribution of the MexX-MexY-oprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **44**, 2242–2246 (2000).
29. N. Mayer-Hamblett, M. Rosenfeld, R. L. Gibson, B. W. Ramsey, H. D. Kulasekara, G. Z. Retsch-Bogart, W. Morgan, D. J. Wolter, C. E. Pope, L. S. Houston, B. R. Kulasekara, U. Khan, J. L. Burns, S. I. Miller, L. R. Hoffman, *Pseudomonas aeruginosa* in vitro phenotypes distinguish cystic fibrosis infection stages and outcomes. *Am. J. Respir. Crit. Care Med.* **190**, 289–297 (2014).
30. D. Hocquet, M. Petitjean, L. Rohmer, B. Valot, H. D. Kulasekara, E. Bedel, X. Bertrand, P. Plésiat, T. Köhler, A. Pantel, M. A. Jacobs, L. R. Hoffman, S. I. Miller, Pyomelanin-producing *Pseudomonas aeruginosa* selected during chronic infections have a large chromosomal deletion which confers resistance to pyocins. *Environ. Microbiol.* **18**, 3482–3493 (2016).
31. G. Cabot, L. Florit-Mendoza, I. Sánchez-Diener, L. Zamorano, A. Oliver, Deciphering β-lactamase-independent β-lactam resistance evolution trajectories in *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* **73**, 3322–3331 (2018).
32. P. Yen, J. A. Papin, History of antibiotic adaptation influences microbial evolutionary dynamics during subsequent treatment. *PLOS Biol.* **15**, e2001586 (2017).
33. G. Cabot, L. Zamorano, B. Moyà, C. Juan, A. Navas, J. Blázquez, A. Oliver, Evolution of *Pseudomonas aeruginosa* antimicrobial resistance and fitness under low and high mutation rates. *Antimicrob. Agents Chemother.* **60**, 1767–1778 (2016).
34. S. J. T. Wardell, A. Rehman, L. W. Martin, C. Winstanley, W. M. Patrick, I. L. Lamont, A large-scale whole-genome comparison shows that experimental evolution in response to antibiotics predicts changes in naturally evolved clinical *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **63**, e0169-19 (2019).
35. M. H. Rau, R. L. Marvig, G. D. Ehrlich, S. Molin, L. Jelsbak, Deletion and acquisition of genomic content during early stage adaptation of *Pseudomonas aeruginosa* to a human host environment. *Environ. Microbiol.* **14**, 2200–2211 (2012).
36. F. Sanz-García, S. Hernando-Amado, J. L. Martínez, Mutational evolution of *Pseudomonas aeruginosa* resistance to ribosome-targeting antibiotics. *Front. Genet.* **9**, 451 (2018).
37. S. M. Cheer, J. Waugh, S. Noble, Inhaled tobramycin (TOBI®): A review of its use in the management of *Pseudomonas aeruginosa* infections in patients with cystic fibrosis. *Drugs* **63**, 2501–2520 (2003).
38. M. L. Workentine, C. D. Sibley, B. Glezerson, S. Purighalla, J. C. Norgaard-Gron, M. D. Parkins, H. R. Rabin, M. G. Surette, Phenotypic heterogeneity of *Pseudomonas aeruginosa* populations in a cystic fibrosis patient. *PLOS ONE* **8**, e60225 (2013).
39. M. Díez-Aguilar, M. I. Morosini, E. Köksal, A. Oliver, M. Ekkelenkamp, R. Cantón, Use of calgary and microfluidic bioflux systems to test the activity of fosfomycin and tobramycin alone and in combination against cystic fibrosis *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* **62**, e01650-17 (2018).
40. G. McCaughey, D. F. Gilpin, T. Schneiders, L. R. Hoffman, M. McKevitt, J. S. Elborn, M. M. Tunney, Fosfomycin and tobramycin in combination downregulate nitrate reductase genes *narG* and *narH*, resulting in increased activity against *Pseudomonas aeruginosa* under anaerobic conditions. *Antimicrob. Agents Chemother.* **57**, 5406–5414 (2013).
41. C. Barbosa, R. Römhild, P. Rosenstiel, H. Schulenburg, Evolutionary stability of collateral sensitivity to antibiotics in the model pathogen *Pseudomonas aeruginosa*. *eLife* **8**, e51481 (2019).
42. T. T. Hoang, R. R. Karkhoff-Schweizer, A. J. Kutchma, H. P. Schweizer, A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: Application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**, 77–86 (1998).

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Rapid and robust evolution of collateral sensitivity in *Pseudomonas aeruginosa* antibiotic-resistant mutants

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DISCUSIÓN

4. Discusión

Como ya se ha explicitado en el prefacio de esta memoria, en las últimas décadas la resistencia a los antibióticos se ha trocado en un problema de tinte global, agudizado por la continua evolución y diseminación de bacterias MDR a lo largo y ancho del planeta [3]. Con el fin de enfrentar adecuadamente este problema, el estudio predictivo de los factores y mecanismos que espolean y/o coercen la evolución de la resistencia se ha revelado como una valiosa fuente de conocimiento, máxime para diseñar racionalmente terapias más efectivas frente a estos microorganismos tan contumaces.

Una de las bacterias Gram-negativas de mayor calado en la multirresistencia es *P. aeruginosa*, tanto por sus elevadas cotas de resistencia intrínseca como por su capacidad para adquirir mutaciones y determinantes de resistencia ubicados en MGEs [21,207], lo que le permite desarrollar resistencia frente a muchos de los antibióticos con los que se tratan las infecciones que causa, inclusive aquellos usados en terapias delimitadas a casos clínicos asaces críticos.

Durante el transcurso de esta tesis, se han llevado a término una serie de experimentos dirigidos al estudio de la resistencia intrínseca y adquirida de *P. aeruginosa*, enfatizando no sólo los mecanismos involucrados, sino también los factores que restringen el proceso evolutivo de la adquisición de resistencia, contribuyendo con todo ello a una potencial mejora en el campo de la predicción de este problema.

4.1. Estudio del resistoma intrínseco a aminoglicósidos de *P. aeruginosa* e identificación de posibles dianas duales de terapias anti-resistencia y anti-virulencia

Esta tesis fue incoada con el estudio del resistoma intrínseco de *P. aeruginosa* frente a aminoglicósidos, mediante el escrutinio de una genoteca de mutantes de inserción de *P. aeruginosa* PA14. Particularmente, se pretendía someter a examen la idea de que el papel de un gen en el resistoma intrínseco frente a una droga, identificado con este método, fuese extensible a todos los restantes adláteres estructurales de la misma, una cuestión todavía en vilo por la exigüidad de estudios al respecto [25,145,146]. Para ello, se determinó la CMI de cuatro aminoglicósidos en una panoplia de mutantes de una genoteca de inserción [265], escogidos a fuer de su constatada implicación en el resistoma intrínseco de esta bacteria a algún aminoglicósido, algo plasmado en dos publicaciones previas, y en base a un escrutinio con amikacina realizado en el laboratorio.

De toda la coalición de mutantes de inserción, sólo dos exhibieron cambios parejos en su sensibilidad a los cuatro aminoglicósidos. Así las cosas, uno no puede sino inferir que, al menos en el caso de los aminoglicósidos, el rol de un gen en el resistoma frente a un determinado antibiótico no debería generalizarse al que posee frente a otros miembros de esta familia química, algo a tener en cuenta en la interpretación de resultados de escrutinios de genotecas de inserción.

Por otra parte, este experimento también supuso el primer reporte sobre dos nuevos genes cuya inactivación incrementaba la sensibilidad de *P. aeruginosa* a algún aminoglicósido, esto es, dos nuevos miembros de su resistoma intrínseco: *glnD* y *mucD*. La pérdida de función del segundo, no obstante, ocasionaba una disminución de la sensibilidad a un aminoglicósido, además de un aumento de ésta frente a otro, lo que avala la conclusión alcanzada en el acápite precedente. Curiosamente, ambos genes han sido propuestos previamente como posibles determinantes de resistencia a β-lactámicos [228,266]. Asimismo, el escrutinio llevado a cabo permitió identificar la potencial implicación de 14 *loci* en la resistencia adquirida a aminoglicósidos. De entre ellos, vale la pena entresacar a *pilC*, ya que el aumento de resistencia originado por su inactivación cubrió los cuatro aminoglicósidos ensayados. El porqué de este fenotipo al inactivar un gen codificante de una proteína participante en la biogénesis de los *pili* de *P. aeruginosa* [267] no ha sido desentrañado en el momento de redactar estos renglones. Ahora bien, se podría aventurar que las modificaciones en la permeabilidad de membrana provocadas por una estructura de *pili* anómala podrían dificultar la entrada de compuestos cargados, en este caso aminoglicósidos, al interior celular, como se ha descrito en el pasado [68]. Ha de aclararse que, si bien la resistencia a aminoglicósidos de *P. aeruginosa* suele estar monopolizada por la adquisición de enzimas inactivantes en muchas circunstancias -especialmente en infecciones agudas [215]-, estos hallazgos sobre resistencia mutacional tienen mayor enjundia en el marco de las infecciones crónicas debidas a esta bacteria [209,210].

La otra meta que se perseguía con este método predictivo era encontrar genes cuya inactivación fuera causante de hipersensibilidad a antibióticos de diferentes familias químicas -además de a los aminoglicósidos-, ya que esto tornaría dichos genes en posibles dianas de coadyuvantes que sensibilizarían a *P. aeruginosa* frente al tratamiento con estos antibióticos. Además, se evaluó si la inactivación de esos *loci* podría ir ligada a una reducción de la virulencia, lo que despuntaría más si cabe la candidatura de los productos de estos genes como blanco de terapias duales anti-resistencia y anti-virulencia.

Dentro de estos requisitos se encuadraron los mutantes de inserción en *PA3016, hpf, PA2798, hflK* y *glnD*. Si bien los mutantes en *hpf, PA2798* y *hflK* mostraban fenotipos bastante prometedores, fue el mutante de inserción en *glnD* el que atrajo más atención. Hasta el momento, se ha documentado que este gen codifica una uridililtransferasa involucrada en el catabolismo de la glicina betaína de *P. aeruginosa* [268], la cual podría desempeñar funciones asociadas al metabolismo del nitrógeno [269,270], mas ningún ligamen con la resistencia a antibióticos o la virulencia se le ha achacado. Sin embargo, el mutante de inserción en *glnD* de este escrutinio desplegó hipersensibilidad a diferentes aminoglicósidos, tigeciclina, tetraciclina, ciprofloxacino y fosfomicina. Por añadidura, su potencial virulento se vio reducido frente al detentado por la cepa PA14. Ante esta tesitura, se podría sacar partido de los efectos pleiotrópicos resultantes de inactivar *glnD*. Primo, la hipersensibilidad a fosfomicina derivada de la inactivación de este gen en *P. aeruginosa* podría explotarse, utilizando un inhibidor de esta proteína

junto a la susodicha fosfomicina, puesto que es uno de los antibióticos de elección para el tratamiento de este patógeno oportunista [245]. De hecho, la tobramicina también podría adherirse a esta terapia, a raíz de su documentada sinergia con la fosfomicina [271,272] y la hipersensibilidad a este aminoglicósido que presenta el mutante de inserción en *glnD*. Secundo, *P. aeruginosa* es intrínsecamente resistente a la tigeciclina [258], pero podría replantearse el uso clínico de este antimicrobiano en compañía de un inhibidor de GlnD, dada la sensibilización a tigeciclina que dicha inhibición podría propiciar. Tertio, este hipotético inhibidor, además de aumentar la sensibilidad a drogas en esta bacteria, también disiparía en gran medida su potencial virulento, allanando el camino para el tratamiento. Ítem más, en la actualidad la búsqueda de compuestos que mermen la virulencia de *P. aeruginosa*, ya sea para emplearse en solitario o sumados a un antibiótico, ha recibido bastante impulso [151,263]. Ahora bien, la aplicabilidad de estas propuestas requeriría del desarrollo de un inhibidor eficaz de esta proteína, que ejerciera el efecto deseado *in vivo* sin incurrir en toxicidad para el paciente.

4.2. La adquisición de resistencia a antibióticos con diana en el ribosoma en *P. aeruginosa* sigue rutas evolutivas limitadas, restringidas por la epistasia y la presión de selección

Tal y como se ha detallado con anterioridad, la resistencia adquirida por las bacterias representa un escollo en el problema global de la resistencia a los antibióticos, más problemático incluso que los mecanismos de resistencia que los patógenos poseen de forma inherente, ya que puede inhabilitar el uso de antibióticos que eran inicialmente útiles en los tratamientos. Por consiguiente, el estudio de la evolución bacteriana en su adaptación a los antibióticos comporta un interés superno. Al hilo de esto, la ALE se ha erigido como una herramienta muy valiosa en la predicción de la resistencia adquirida por mutaciones, tanto en presencia de antibióticos actualmente utilizados en clínica, como de agentes antimicrobianos en incipiente desarrollo [273-275].

Durante el ínterin que ha abarcado esta tesis, se ha hecho uso de esta aproximación experimental con *P. aeruginosa* en presencia de distintos antibióticos. En primer lugar, en concentraciones crecientes de dos antibióticos cuya diana es el ribosoma: tobramicina y tigeciclina. La tobramicina constituye un fármaco de elección en las infecciones por *P. aeruginosa* [243,244]; mientras que la tigeciclina, a pesar de no aplicarse en clínica para tratar estas infecciones -al ser este patógeno intrínsecamente resistente a ella [258]-, sí puede ejercer presión selectiva sobre *P. aeruginosa* cuando ésta forma parte de superinfecciones en pacientes previamente tratados con tigeciclina [255], y en ecosistemas naturales contaminados con bajas concentraciones de este compuesto.

Más allá de la importancia que entrañaba descombrar el sendero mutacional que dotara de resistencia a estos antimicrobianos, una de las finalidades de estas ALEs radicaba en la comparación entre los mecanismos de resistencia seleccionados frente a dos drogas que inhiben, de forma dispar, un mismo blanco; lo que nos llevó a identificar las mutaciones en *orfN* y *pmrB* como las únicas compartidas entre las dos evoluciones

adaptativas. Los vínculos previos de OrfN con la resistencia a ciprofloxacino [276], aztreonam [277] o a la propia tobramicina [278] inciden en un posible papel de esta glicosil transferasa en alteraciones generales de la permeabilidad de membrana. Es de destacar que tanto estas mutaciones previamente descritas como las obtenidas en este estudio ocurren en regiones repetidas poli-G, por lo que se colige que este gen podría atesorar una elevada frecuencia de mutación como consecuencia del deslizamiento de la polimerasa -al menos en PA14, ya que *orfN* es altamente polimórfico en *P. aeruginosa*- [279,280], y eso explicaría su infalible selección temprana en todas las poblaciones de estas ALEs. A tenor de *pmrB*, este gen codifica un miembro del sistema de dos componentes PmrAB, cuya función reguladora afecta al LPS bacteriano y, por tanto, su mutación puede desembocar en resistencia a péptidos catiónicos y variabilidad en la sensibilidad a quinolonas, tigeciclina y tobramicina, entre otros [281-284].

En lo que a la ALE en tobramicina concierne, las mutaciones recurrentes y primeramente seleccionadas por antonomasia, obviando la presente en *orfN*, fueron las localizadas en *fusA*. Mutaciones en el factor de elongación G que codifica este gen ya han sido propuestas en el pasado reciente como primera respuesta a los aminoglicósidos [155,156,186,278,285], lo que nuestros resultados no hacen sino ratificar. Por su parte, *ptsP* presentó mutaciones en la mitad de las poblaciones evolucionadas en presencia de tobramicina, algo que se correlaciona con la resistencia a este antibiótico que exhibió su mutante de inserción en el escrutinio que principió esta tesis, o las evidencias que existen sobre la selección de mutantes en *ptsP* frente a aminoglicósidos [132], más particularmente tobramicina [25,278,286]. Llegados a este punto, conviene hacer un alto para quintaesenciar estos resultados: según lo recogido en el estudio del resistoma intrínseco a tobramicina de *P. aeruginosa* en [25], existe un mínimo de 135 genes cuya inactivación reduce la sensibilidad bacteriana a esta droga; y eso sin considerar los 14 descritos en esta tesis, ni aquellos que auparían la resistencia vía mutaciones de ganancia de función o de inactivación parcial. A resultas de estos guarismos, las posibles permutaciones de mutaciones que condujeran a resistencia a tobramicina en esta bacteria, tomadas de 5 en 5 (el número máximo acumulado en una réplica poblacional de nuestra ALE), frisan cantidades tan astronómicas -4.2E10, si se obvia la posibilidad de varias mutaciones en un mismo gen- que la evolución adaptativa habría de ser, por fuerza, impredecible. Sin embargo, nuestro análisis expone las mutaciones en *orfN* como las seleccionadas en primer lugar invariablemente en todas las poblaciones, seguidas de una igualmente conservada adquisición de mutaciones en *fusA* y *pmrB*. En virtud de tales resultados, se puede asertar que las trayectorias evolutivas de *P. aeruginosa* hacia la resistencia a tobramicina gozan de cierto grado de predictibilidad. Este sesgo determinista resulta harto llamativo al discordar con la naturaleza estocástica de la evolución, y es debido a la existencia de factores que restringen las rutas evolutivas posibles bajo condiciones concretas. Así, la estocasticidad de la evolución no debería ser considerada un axioma, al menos en el caso de la resistencia a los antibióticos.

Viremos hacia la tigeciclina: para empezar, se ha de valorar que *P. aeruginosa* desarrolló altos niveles de resistencia a esta glicilciclina durante la ALE, aun cuando ya

presumía de resistir intrínsecamente a la misma, clínicamente hablando. Por si fuera poco, la adaptación a ella desplegó una patulea de resistencias cruzadas a antibióticos clínicamente relevantes, por lo que advertimos que el uso de tigeciclina en pacientes que puedan ser posteriormente super-infectados por este patógeno oportunista podría conllevar consecuencias alarmantes. En lo que respecta a las mutaciones seleccionadas, el número y variedad de éstas fue netamente superior al que se dio en la ALE con tobramicina. Aparte del omnipresente *orfN*¸ el gen de más pronta mutación en todas las réplicas fue *nfxB*, correspondiente al represor local de la expresión de la bomba de expulsión MexCD-OprJ [287]. Estas mutaciones, como se comprobó, provocaron la sobre-expresión de este sistema, por lo que MexCD-OprJ puede haber contribuido al fenotipo de resistencia frente al antibiótico de selección y otras drogas [288]. Además, se seleccionaron mutaciones en las subunidades que conforman la bomba, que podrían ser mutaciones compensatorias del coste de *fitness* que a menudo conlleva el sobre-expresar estos mecanismos [115]. Tampoco es descabellado colegir que mejoren la especificidad por el sustrato, dado que el polimorfismo seleccionado en *mexD* propicia un cambio de aminoácido en una región periplásmica a la que compete precisamente el reconocimiento de la molécula que expele [289]. Nótese que, a pesar de no ser un caso tan cristalino como el de la tobramicina, la evolución de la resistencia a tigeciclina de las poblaciones analizadas también propendió hacia la convergencia, lo que anuncia un limitado número de trayectorias evolutivas hacia dicha resistencia, en las condiciones estudiadas. De esta manera, los resultados discutidos apuntan a que la evolución de la resistencia vía mutación frente a antibióticos con diana en el ribosoma en *P. aeruginosa* tiene cierto matiz de predictibilidad, al menos cuando las bacterias crecen en las mismas condiciones. Esta aportación podría mejorar el cariz de la predicción de la resistencia adquirida por parte de este conspicuo patógeno.

Durante estas ALEs, una deleción de dos pares de bases -referida a partir de ahora como mutación de pérdida de función, dado su efecto- en el gen *lasR*, que codifica uno de los reguladores maestros de la respuesta QS [290], se seleccionó en todas las poblaciones de *P. aeruginosa* PA14 evolucionadas en medio sin antibiótico, lo que sugería una posible mejora del *fitness* en dicho medio. Empero, ninguna de las poblaciones que evolucionaron en presencia de antibiótico preservó esta mutación en etapas finales de las ALEs, a pesar de haberse detectado en sus primeros pasos. Esto llevó a hipotetizar que esta mutación en *lasR* ejercía un efecto epistático negativo sobre una o varias de las mutaciones de resistencia que fueron seleccionadas en presencia de antibiótico.

Es sabido que la epistasia es uno de los factores que condicionan la evolución de la resistencia a antibióticos en bacterias. Su influencia se puede manifestar en el orden de adquisición de mutaciones de resistencia, ya que una primera mutación puede restringir la selección de las venideras, situación conocida como contingencia [183]. Aunque este fenómeno ha recibido bastante atención en el pasado reciente [109,133,186,291], los estudios que lo abordan han buscado, mayormente, desentrañar las interacciones epistáticas entre genes y mutaciones de resistencia, haciendo caso omiso a las relaciones que pueden establecerse entre estas últimas y otras mutaciones que posibiliten, mediante una mejora

del *fitness* bacteriano, la adaptación a un nuevo hábitat (i. e., el hospedador). En consecuencia, en esta tesis se emprendió este sendero, tomando como mutación de adaptación al ambiente la antedicha en el gen *lasR*. Es de resaltar que mutantes defectivos en este regulador han sido aislados de un alto porcentaje de pacientes aquejados de infecciones crónicas [292,293], lo que se ha achacado a una optimización del proceso infectivo causada por el aventajado crecimiento de los mutantes *lasR* en estas condiciones [294,295]. Así, en primera instancia, se caracterizó el mutante en *lasR* obtenido mediante ALE, confirmando que era defectuoso en los fenotipos relacionados con QS. Es de interés reseñar que dicho mutante presentó una mejora del *fitness* respecto a su cepa parental, alcanzando una mayor densidad óptica que ésta, creciendo en medio exento de antibiótico. A sabiendas de que la desactivación del QS conlleva a su vez un ahorro energético [296], es entendible la frecuencia con la que los genes codificantes de reguladores de QS adquieren mutaciones inactivantes durante ALEs, algo no solamente ocurrido en nuestro ensayo [297,298]. Por otra parte, no se apreciaron diferencias entre las CMIs de tobramicina y tigeciclina del mutante en *lasR* y la cepa silvestre, por lo que se descartó la posibilidad de que la mutación en ese gen no se seleccionara por incrementar la sensibilidad a estos antimicrobianos.

Seguidamente, se realizó una ALE del mutante en *lasR*, de idénticas características a la pergeñada con *P. aeruginosa* PA14, con vistas a comparar las trayectorias evolutivas, a nivel genotípico y fenotípico, seguidas por esta cepa y la parental. En primer término, se advirtieron disimilitudes en el nivel de resistencia adquirida a los antibióticos de selección, si se parangonaban con lo catalogado en la cepa silvestre. Esta discrepancia se observó también en los tipos y niveles de resistencia cruzada y sensibilidad colateral. Por tanto, todo ello aludía a la existencia de relaciones epistáticas entre la mutación en *lasR* y mutaciones de resistencia, que modificaban la hechura de las trayectorias evolutivas y el fenotipo resultante bajo la selección de los antibióticos empleados.

En cuanto al genotipo, éste difirió nuevamente entre ambos fondos genéticos. Si bien algunos de los genes cuyas mutaciones fueron presuntamente responsables de la resistencia a los antibióticos de selección en PA14 sufrieron mutaciones a su vez en el mutante *lasR* (*fusA* y *ptsP* en tobramicina; *nfxB* en tigeciclina; *orfN* y *pmrB* en ambos antibióticos), también se identificaron mutaciones surgidas en un fondo genético, pero ausentes en el otro. En lo que a la ALE en tobramicina concierne, la balanza se inclinó hacia una mayor variedad de mutaciones nuevas en el contexto *lasR*. Entre ellas destacan los cambios genéticos en *orfH* y *orfK*, que precisamente se hallaron en las poblaciones carentes de mutaciones en *orfN*; por lo que, dado que colocalizan en la misma isla genómica de glicosilación de flagelinas [280], sería razonable suponer que su función en la adquisición de resistencia sea similar a la que puede mediar *orfN* [278]. La ALE del mutante en *lasR* en presencia de tobramicina seleccionó también mutaciones en *mexZ*, *rpsL*, *tonB* y *nuoD*, siendo todos ellos genes con precedentes en la resistencia a antibióticos [25,299-301]. El caso de la tigeciclina es más variopinto: algunas de las mutaciones seleccionadas en PA14 no se seleccionaron en la cepa defectiva en *lasR* y, a la inversa, tres nuevas mutaciones surgieron en el contexto defectivo en QS: en los

genes *phoQ*, *rpoA* y *mutL*. Esta última merece capítulo aparte: una mutación en *mutL* fue seleccionada en una población *lasR* al inicio de la evolución en presencia de tigeciclina y, a razón de codificar un componente del sistema de reparación de errores de replicación [302], era de esperar que produjera un fenotipo hipermutador [303]. De acuerdo con ello, la población contenía un número visiblemente superior de mutaciones (fundamentalmente transiciones), la mayoría de las cuales no tenían que estar necesariamente conectadas con la resistencia. Por otro lado, PhoP-PhoQ es un sistema de dos componentes cuyo nexo con la resistencia a aminoglicósidos y polimixina B [304,305], amén de a tetraciclina -a través de la regulación de la expresión de un sistema de transporte [306]- ha sido documentado. En cambio, *rpoA* codifica la subunidad α de la ARN polimerasa, y mutaciones en este gen han sido adjudicadas tanto a la adquisición de resistencia (a quinolonas, en *S. enterica*) [307] como a la compensación del coste de *fitness* suscitado por la obtención de resistencia (a rifampicina, en *M. tuberculosis*) [308]. Discernir el rol que están jugando las mutaciones de *rpoA* en nuestro caso exigiría más investigación.

Hasta el momento, todos los resultados apuntaban a la existencia de epistasia negativa entre la mutación en *lasR* y mutaciones de resistencia; dicho de otra forma, el alelo *lasR* mutante podría reducir el *fitness* en presencia de antibiótico en mutantes resistentes a tobramicina o tigeciclina. No obstante, tal hipótesis debía ser probada. Para ello, se idearon dos maniobras: la introducción de la mutación de *lasR* en dos mutantes de tiempo final, que contenían un conjunto de mutaciones representativas de las poblaciones evolucionadas de la cepa silvestre en uno u otro antibiótico; y en un mutante de tiempo inicial en el gen *orfN*, que se seleccionó de forma temprana en ambas ALEs. Con la primera aproximación se observó que, en un clon aislado de una población final evolucionada en presencia de tobramicina, la inclusión del alelo mutante de *lasR* acrecía la sensibilidad a ésta, medida como CMI; y que en un clon aislado de una población evolucionada en tigeciclina, dicha inclusión aminoraba su *fitness*, medido como crecimiento en presencia de este antibiótico (su CMI rebasaba el límite de detección del E-test). Por si fuera poco, la resistencia cruzada y la sensibilidad colateral a otros antibióticos que estos clones presentaban variaba considerablemente respecto a sus correspondientes cepas parentales. Todo ello apoyaba la idea de la epistasia negativa entre mutaciones en este regulador de QS y mutaciones de resistencia. Por su parte, la segunda aproximación permitió entender los primeros estadios de ambas evoluciones, necesarios para establecer la contingencia que encorsetó las trayectorias evolutivas diferenciales. Con este fin se aisló un clon con la mutación en *orfN*, presente en todas las réplicas de la evolución en PA14 tras los 5 primeros días, y en 5 de 8 poblaciones en la estirpe defectiva en *lasR*. Si la hipótesis de la epistasia negativa fuera válida, la introducción de la mutación en *lasR* en el mutante en *orfN* debería menguar su *fitness*, medido como crecimiento bajo presión de selección. Hecho el experimento, esa hipótesis fue corroborada. Es decir, el crecimiento en tobramicina o tigeciclina se veía negativamente afectado en el doble mutante en comparación al mutante en *orfN*, ergo la epistasia existente entre mutaciones tempranas en *orfN* y *lasR* desembocó en que las primeras prevalecieron sobre las segundas en presencia de antibiótico.

En conclusión, con este experimento se ha examinado prolijamente uno de los elementos con papel prominente en la evolución de la resistencia a antibióticos, que es la epistasia. Si bien ésta ha sido previamente analizada entre mutaciones de resistencia [180,291], en esta tesis se ha determinado la interacción epistática recíproca que existe entre un regulador de virulencia (en particular, de la respuesta QS) y mutaciones de resistencia a antimicrobianos con diana en el ribosoma en *P. aeruginosa*. Así, se ha observado que las trayectorias conducentes a dicha resistencia son contingentes con mutaciones previas de adaptación al ambiente, relación que es además bilateral. Hilando más fino todavía: mutaciones eco-adaptativas en *lasR* seleccionadas en ausencia de presión selectiva coartan el tipo de mutaciones y nivel de resistencia que se pueden seleccionar una vez aplicada la presión. Y viceversa: mutantes *lasR* defectivos en QS ven vetada su selección cuando determinadas mutaciones de resistencia se seleccionan con anterioridad, al menos en las condiciones experimentales estudiadas. Así, resulta notable que la mutación de un único gen (*a priori* no implicado en resistencia) trastoque completamente las singladuras evolutivas hacia la resistencia y las redes de resistencia cruzada/sensibilidad colateral, encumbrando de este modo la influencia de la epistasia en este ámbito. Todo ello constituye información de peso que manifiesta la dificultad que reviste la predicción de la evolución de la resistencia a antibióticos en fondos genéticos variados de este patógeno -situación próxima a la realidad *in vivo*-, a la par que establece por vez primera una relación epistática entre elementos clave de la virulencia y la resistencia de *P. aeruginosa*, dos de sus estiletes capitales en el proceso infectivo.

A continuación, se procedió a completar el estudio de la resistencia adquirida a antibióticos con diana en el ribosoma en *P. aeruginosa*, desde el prisma de diferentes presiones de selección, específicamente concentraciones inferiores a la letal. Hoy día se sabe que presiones selectivas leves, i. e. concentraciones subletales de antibiótico, pueden seleccionar mutantes resistentes [173], lo que expande el rango de hábitats en los que la resistencia bacteriana a antibióticos puede emerger, antaño circunscrito esencialmente a hospitales, granjas, piscifactorías y otros ambientes con alta contaminación por estos compuestos. Esta preocupante tesitura es contemplada dentro de la perspectiva *One Health* [6], desde la que se pretende lidiar actualmente contra la resistencia a antibióticos y que se vería muy beneficiada de ahondar en el conocimiento de las ventanas de selección de antibióticos de utilidad clínica y los fenotipos de resistencia cruzada que originan. Tal es la información que se ha obtenido para tobramicina y tigeciclina en esta tesis, sirviéndonos de ALEs en presencia de concentraciones subletales constantes de estas drogas. Los valores escogidos fueron 1/10 y 1/50 de la CMI de la cepa silvestre, en aras de abarcar una ventana amplia que permitiera estudiar la selección de mutantes resistentes incluso en concentraciones muy bajas de fármaco. Al mismo tiempo, la comparación de estos resultados con los de la ALE antes descrita en concentraciones letales crecientes de estos mismos antimicrobianos, revelaría el papel que juega la presión de selección en la evolución de la resistencia a tobramicina y tigeciclina en *P. aeruginosa*.

La primera observación que se extrajo del estudio fue que las dos concentraciones subletales de tigeciclina testadas seleccionaron mutantes resistentes, y no así las de tobramicina. Este hecho es trascendente por sí solo, ya que se han descrito otros antimicrobianos, p. ej. ciprofloxacino, que seleccionan resistencia en concentraciones ínfimas (1/230 de la CMI de la cepa silvestre) [173]. Es más, es sabido que la tobramicina selecciona resistencia a concentraciones de 1/2 de la CMI de la cepa silvestre [178]. En nuestro ensayo, 1/10 de la CMI de PA14 fue insuficiente para dicha selección, lo que se ve justificado a razón del parco efecto que ejercía esta concentración de tobramicina en el crecimiento de la bacteria -a diferencia del causado por la tigeciclina-. De hecho, se sabe que el *fitness* en presencia del antibiótico de selección es el elemento clave que promueve la selección de mutantes resistentes enfrentados a concentraciones subletales del mismo [309]. En resumen, este resultado subraya que la ventana de selección de resistencia es específica de cada antibiótico, siendo la de la tigeciclina manifiestamente más amplia que la de la tobramicina. En vista de ello, sería interesante que cada antibiótico fuese analizado individualmente, en lo que a su rol como selector de resistencia respecta, para predecir el efecto que pueda ocasionar, especialmente en los ecosistemas naturales donde exista contaminación por estos compuestos.

El caso de la tigeciclina es más escabroso. Concentraciones subletales de esta glicilciclina dieron lugar a una menor resistencia a la susodicha en comparanza a las letales; empero, algunas resistencias cruzadas surgidas tras la ALE subletal fueron preocupantemente más acentuadas que las seleccionadas en concentraciones letales, algo que contraviene trabajos previos [119]. Por ejemplo, la resistencia a levofloxacino -usado en el tratamiento de *P. aeruginosa* [310]-, fue superior en las réplicas evolucionadas en concentraciones subletales de tigeciclina. A la vista de esto, se puede afirmar que la resistencia cruzada de *P. aeruginosa* asociada a la adquisición de resistencia a tigeciclina es dependiente del grado de presión de selección aplicado durante la ALE. Asimismo, se ha de insistir en la importancia de que concentraciones tan reducidas como 1/50 de la CMI de tigeciclina sean capaces de seleccionar fenotipos de elevada resistencia a otros antibióticos clínicamente útiles, como las quinolonas. Aunque en la órbita nosocomial sólo cabe esperar que la glicilciclina ejerza presión selectiva sobre *P. aeruginosa* en pacientes super-infectados [255], es dable que concentraciones subletales de ésta contaminen hábitats que este patógeno coloniza allende la clínica, pudiendo haber sido vertida al medio ambiente tras su uso hospitalario [177,311]. Dado que este hecho puede propiciar la selección de multirresistencias, la información derivada de este estudio puede ser de utilidad en tanto en cuanto la perspectiva *One Health* pavimenta el proceder actual en la lid contra la resistencia a antibióticos [6,312].

Seguidamente, se profundizó en las mutaciones presentes en las poblaciones evolucionadas en condiciones subletales de tigeciclina. Entre ellas, las más numerosas surgieron en genes codificantes de proteínas relacionadas con el flagelo: *fleQ*, *fleRS* y *flgE*. Pese a que en el primero también se seleccionó una mutación en una de las poblaciones evolucionadas en concentraciones letales de tobramicina, en general el vínculo de estos genes con la resistencia a antibióticos es, según lo sabido a día de hoy,

laxo, y en ningún caso relacionado con la tigeciclina [146,313]. Lo contrario ocurre con *pilQ2*, gen que codifica una proteína de *pilus* tipo IV B y cuyas mutaciones trastocan la permeabilidad de membrana, remitiendo la entrada de antibióticos hidrofílicos como la tigeciclina [68]. Mutaciones en *pilQ2* han sido identificadas en aislados de *P. aeruginosa* procedentes de pacientes con FQ [69], o en presencia de concentraciones subletales de ciprofloxacino [314].

Una vez glosadas las mutaciones de mayor primacía presentes al final de la ALE en concentraciones subletales de tigeciclina, vuelve a imponerse la comparación con la evolución llevada a cabo bajo presión de selección letal. Para empezar, el repertorio de mutaciones en este último caso fue considerablemente mayor y más variado, en consonancia con lo publicado [168], aunque no con lo teóricamente esperable [315]. Esta aparente disparidad deviene probablemente de la desemejanza de los diseños experimentales: concentraciones subletales de antibiótico mantenidas en el tiempo tienden a seleccionar sólo los mutantes más competitivos, mientras que el aumento escalonado de concentraciones letales favorece la acumulación secuencial de mutaciones que confieren un elevado nivel de resistencia. Por el mismo motivo, no es de extrañar que la evolución en condiciones letales condujera a la aparición de mutaciones de afamada prosapia en resistencia (en los genes *nfxB*, *mexCD*, *parRS*, *rpsJ* o *pmrB*) y no así la consumada en condiciones subletales. El único gen mutado coincidente entrambas fue *orfN*, si bien sólo se vio alterado en una de las poblaciones evolucionadas en concentraciones subletales, como contrapunto a su recurrencia en todas las contrapartidas letales. A la luz de todo lo antedicho, se puede concluir que la presión de selección es un factor harto influyente en la evolución de la resistencia a tigeciclina en *P. aeruginosa*. Además, en base a estos resultados, se puede predecir hasta cierto punto el nivel de resistencia, resistencia cruzada y sensibilidad colateral que esta bacteria alcanzaría en presencia de determinadas concentraciones de esta droga.

A modo de epítome, puede afirmarse que estas tres ALEs, jalonadas en sendos artículos, nos han proporcionado un valioso conocimiento predictivo sobre los potenciales mecanismos de resistencia -algunos ya celebérrimos y otros descritos por vez primera- que se pueden seleccionar cuando *P. aeruginosa* es tratada con uno de sus antibióticos de elección más en boga, como es la tobramicina, y la baja probabilidad de seleccionar resistencia en concentraciones bajas de la misma. También nos han informado sobre la selección de resistencia cruzada frente a drogas clínicamente relevantes en este patógeno, tras ser sometido a distintas presiones de selección de tigeciclina; y sobre el rol taxativo de la epistasia en la evolución de la resistencia a ambos antimicrobianos.

4.3. La evolución de la resistencia a ceftazidima y ceftazidima-avibactam en *P. aeruginosa* pivota sobre grandes deleciones cromosómicas, que conllevan una sensibilidad colateral robusta en mutantes resistentes

La combinación ceftazidima-avibactam es una terapia dual emergente cuya celosa restricción a casos clínicos extremos hace tanto más interesante el análisis de sus

futuribles mecanismos de resistencia [252]. Pese a que existe información exhaustiva acerca de diferentes determinantes de resistencia adquiridos por HGT contra parejas tradicionales de β-lactámico-inhibidor de β-lactamasas [316,317], que posiblemente podrían servir también de égida a *P. aeruginosa* frente a ceftazidima-avibactam, poco se sabe actualmente sobre la resistencia conferida por mutaciones frente a este tratamiento bisoño [254,318], una vía evolutiva particularmente preeminente en las infecciones crónicas causadas por este patógeno [210]. Por consiguiente, en esta tesis se recurrió de nuevo a los ensayos de ALE, en esta ocasión en presencia de ceftazidima y su combinación con avibactam, a fin de identificar posibles mecanismos de resistencia por mutación que se podrían seleccionar en *P. aeruginosa* para contrarrestar la acción de esta pareja novel de fármacos, en comparación a los que priman frente a la ceftazidima en solitario.

En ambos casos, el primer evento genético que acaeció en la evolución fue la deleción de vastas regiones del cromosoma bacteriano que incluían, entre otros, los genes *hmgA*, *galU* y *mexXY*. Este genotipo estaba asociado a un fenotipo piomelanogénico, a la par que a un aumento en la resistencia a ceftazidima e hipersensibilidad a amikacina. Este fenómeno ha sido advertido en ensayos de ALE en presencia de la propia ceftazidima [231], y de otros β-lactámicos como el meropenem [160] y la piperacilina [286]; todos ellos avalando la pérdida de *galU* como el principal artífice de la resistencia a esas drogas, dada la participación de este gen en la síntesis del LPS [228]. De hecho, este evento también se ha hallado con cierta frecuencia (13%) en aislados clínicos, algo que se ha ligado a una mayor resistencia al estrés oxidativo y a la persistencia de *P. aeruginosa* en infecciones crónicas pulmonares [241,242,319]. Así, nuestros resultados subrayan la importancia que este cambio genético puede tener en la resistencia a ceftazidima, aun cuando ésta se utilizase con inhibidores de beta-lactamasas.

Además de las grandes deleciones cromosómicas, otro mecanismo imperante seleccionado en ambas ALEs, en presencia o no del inhibidor, fueron las mutaciones en genes codificantes de reguladores y componentes de bombas de expulsión múltiple de drogas: MexAB-OprM y sus dos represores de mayor ascendencia -MexR y NalD- [52,224], y una bomba no descrita (PA14_45890-PA14_45910) y su teórico regulador -un sistema de dos componentes: PA14_45870-PA14_45880-. Huelga decir que la actividad de estos sistemas de bombeo es la explicación más razonable al fenotipo de resistencia cruzada a antimicrobianos de otras familias que se detectó en todas las poblaciones evolucionadas; situación alarmante sobremanera si se extrapolara a la clínica, algo probable en el marco de las infecciones crónicas, donde las mutaciones son el motor principal de la resistencia [210]. Ahora bien, aunque la función de MexAB-OprM en la resistencia a ceftazidima y otros β-lactámicos es archiconocida [288], el rango de sustratos de la bomba putativa es todavía desconocido. Un dato indicativo, eso sí, sería la exuberante resistencia a imipenem que mostraron las poblaciones poseedoras de mutaciones en los genes del sistema de dos componentes y de la bomba PA14_45890-PA14_45910, lo que apunta a que este sistema RND puede poseer cierto grado de especificidad hacia este fármaco. No obstante, tal sospecha habrá de ser clarificada en investigaciones posteriores. Al hilo de la laya de mutaciones en los genes que codifican

las propias bombas, conviene mencionar que la reincidente selección de polimorfismos en las subunidades de estos sistemas (MexB y PA14_45890) podría responder nuevamente a una compensación del coste de *fitness* asociado a su sobre-expresión, o a los, por ahora, incipientemente estudiados casos de mejora en la especificidad del sistema, dando lugar, en este caso, a una expulsión más eficiente de la ceftazidima [55-57]. Se trataría de una dicotomía similar a la planteada por los polimorfismos en las subunidades de MexCD-OprJ seleccionados en la ALE en tigeciclina. Independientemente de su aún inextricable función, es interesante recalcar que mutaciones en *mexB* se han identificado también escaso tiempo ha en cepas clínicas [320]. Como colofón a lo concerniente a las bombas, es de recibo plasmar el posible nexo existente entre la selección de las antecitadas deleciones cromosómicas y una actividad más eficiente de MexAB-OprM. Esto es así porque las bombas MexXY y MexAB comparten (y, en consecuencia, compiten por) la porina OprM, lo que produce antagonismo si los dos sistemas se hallan altamente expresados [321]. Por ende, la eliminación de *mexXY*, sita en las grandes deleciones, podría favorecer el desempeño de MexAB-OprM en su labor de extrusión de ceftazidima. Nótese también que la ausencia de MexXY-OprM explica nítidamente la mayor sensibilidad a amikacina observada en las poblaciones evolucionadas, dado que es una bomba capaz de expulsar aminoglicósidos [220].

En la vertiente de la ALE con ceftazidima en solitario, llaman la atención las mutaciones seleccionadas en elementos implicados en la regulación indirecta de AmpC (*dacB* y *mpl*), que disparan la actividad β-lactamasa de las poblaciones que las presentan. Es menester remarcar que esto no ocurre en presencia de ceftazidima-avibactam, lo que sugiere que el avibactam parece inhibir eficazmente su diana AmpC, al menos en el caso de *P. aeruginosa* PA14. Eso sí, estudios pretéritos desmienten que la inacción de esta β-lactamasa frente este tratamiento combinado se cumpla siempre a rajatabla en otras cepas, y menos si éstas son previamente resistentes a ceftazidima [322]. En nuestro caso, las citadas grandes deleciones y los sistemas de expulsión parecen detentar una posición privilegiada como los mecanismos más prontamente seleccionados en *P. aeruginosa* en presencia de la combinación ceftazidima-avibactam, tornando así predecibles, en cierto grado, las rutas evolutivas hacia su resistencia. La imprevista conservación del primer mecanismo, fácilmente reconocible por la ausencia de *mexXY*, que ocurre tempranamente (el primer día de evolución adaptativa) tras exponer la bacteria a ceftazidima -acompañada o no de avibactam-, nos lleva a ponderar su validez como marcador genético indicativo de hipersensibilidad a aminoglicósidos; siendo éste un dato halagüeño que hemos explorado y será detallado posteriormente. Así, a grandes rasgos, estas ALEs, en connivencia con estudios anteriores, han desvelado una posible trabazón entre la resistencia adquirida a antibióticos y la persistencia de *P. aeruginosa* en enfermos de FQ -dado el papel propuesto para la piomelanina en la perduración de infecciones crónicas [242]-, aunque sería preciso un análisis más profundo para calibrar el alcance de estas conclusiones.

A pesar de que las ALEs anteriores ya dejaron constancia del sustancial potencial predictivo que alberga esta metodología con respecto a mutaciones de entidad clínica, es menester detenerse en este caso concreto. Además de la adquisición de mutaciones en determinantes de resistencia cuya incidencia en clínica es ya trillada, i. e. MexAB-OprM [284,320,323-325], algunas de las mutaciones seleccionadas en nuestro experimento coinciden exactamente con las registradas en aislados clínicos. Es el caso de R504C o R504H en *ftsI* [142,231,326], T11N en *nalD* [327] y V124G en *mpl* (en el aislado de *P. aeruginosa* NCGM1984). Por si fuera poco, un estudio reciente de cepas resistentes a ceftazidima-avibactam aisladas de pacientes ha confirmado la presencia de mutaciones en los mismos genes que detectamos mediante ALE *in vitro*, incluso aquellos cuyo vínculo con la resistencia no era paladino y podrían adscribirse a compensaciones de *fitness*: *dnaK* y *ctpA* como los casos más predominantes, pero también *flgF*, *glnD*, *pcm*, *spoT*, *dnaJ* o el operón codificante de la mencionada bomba de expulsión PA14_45890-PA14_45910 [266]. Por todo ello, la ALE se refrenda como un método de predicción de resistencia adquirida de considerable validez en su extrapolación al paciente.

De todos los resultados obtenidos en las recién discutidas ALEs de *P. aeruginosa* PA14 en presencia de ceftazidima y ceftazidima-avibactam, un fenómeno llamó especialmente nuestra atención: la selección, en el primer paso en todas las réplicas, de grandes deleciones cromosómicas aparejadas a hiper-producción de piomelanina y sensibilidad colateral a amikacina, un evento que también se había catalogado en otras cepas de *P. aeruginosa* evolucionadas en presencia de distintos β-lactámicos [158,160,231] e incluso con bastante asiduidad en aislados de pacientes con FQ [241,242]. Este bagaje tornaba plausible intuir una posible conservación de la selección de deleciones de estas regiones cromosómicas en *P. aeruginosa* al ser enfrentada a β-lactámicos y, de darse esta conservación, también se mantendría la sensibilidad colateral a amikacina (o tobramicina, otro aminoglicósido clínicamente importante), algo a tener en consideración. Nótese que la sensibilidad colateral a un antimicrobiano surgida de la adquisición de resistencia a otro es un *trade-off* evolutivo que reviste mucho interés, ya que podría sacársele partido en el diseño de tratamientos que atajaran casos clínicos agravados por la resistencia a antibióticos [123,127,130,132,328]. Sin embargo, la aplicación de este concepto adolece todavía de validez a raíz de la nula certitud de conservación de la sensibilidad colateral que existe entre distintos contextos genéticos de una misma cepa bacteriana, ya que la sensibilidad puede ser contingente con las rutas evolutivas hacia la resistencia al primer antibiótico que se administra [329]. Dado que existían evidencias que apuntaban a la conservación en nuestro caso, se decidió poner a prueba esta hipótesis contrastando el grado de robustez del supradicho dúo gran deleción/sensibilidad colateral a tobramicina en una colección de mutantes resistentes a distintas drogas, con la aspiración de que la administración de ceftazidima seleccionara el mismo evento evolutivo [243] en un sustancial número de fondos genéticos. Recientemente se ha constatado que las mutaciones de resistencia correlacionan con patrones de sensibilidad colateral robustos o errátiles según alteren la diana del antimicrobiano o elementos reguladores, respectivamente [133]. En consecuencia, la designación de los mutantes construidos para este experimento tuvo como finalidad la máxima heterogeneidad de

resistencias y funciones de los genes mutados: unos contenían mutaciones en proteínas diana, otros en reguladores, y otros en ambas categorías.

Proveídos de estas herramientas, se realizó una evolución corta en ceftazidima. Tras un día de ALE, se observó un nivel de robustez significativo en el comportamiento de los mutantes y réplicas ensayadas: 27 de 32 poblaciones sufrieron deleciones (que incluían *mexXY*), y presentaron un fenotipo piomelanogénico y sensibilidad colateral a tobramicina. Este resultado sugiere que las deleciones cromosómicas abarcando *mexXY* son seleccionadas consistentemente como primer paso en la evolución de la resistencia a ceftazidima en *P. aeruginosa* PA14, al menos en los fondos genéticos ensayados. Si bien es cierto que todavía queda un remanente de impredecibilidad, la convergencia fenotípica de *P. aeruginosa* PA14 hacia la sensibilidad colateral a tobramicina cuando evoluciona en presencia de ceftazidima es notablemente robusta, algo de lo que se podría sacar provecho a título clínico -lo cual requeriría medir el grado de conservación de este evento en cepas hospitalarias-. Eso sí, la envergadura de este hallazgo no parece trivial: los propios resultados albergados en esta tesis han atestiguado la dificultad que esconde el hallar un patrón evolutivo común hacia la resistencia a antibióticos en dispares fondos genéticos, ya que la pérdida de función de un único gen (*lasR*) subvertía completamente dichos patrones, aun tratándose de la misma cepa bacteriana.

Llegados a este punto, se planteó la siguiente disyuntiva: siendo conscientes de la heteróclita variedad poblacional que caracteriza a *P. aeruginosa* en los pulmones de un paciente con FQ (que a veces engloba clones piomelanogénicos resistentes a ceftazidima e hipersensibles a tobramicina) [241,319,330], la eventual explotación de la sensibilidad colateral a tobramicina podría no ser tal. Por ello, se columbró una terapia alternada de tobramicina y ceftazidima, que pudiera conducir certeramente a hipersensibilidad a la primera en este escenario heterogéneo recreado *in vitro*.

Así pues, se conformaron poblaciones heterogéneas a partir de la mixtura entre un clon piomelanogénico correspondiente a cada mutante resistente, y su pertinente cepa parental, a fin de emular la situación mentada. Una evolución de tres días en tobramicina desembocó en la eliminación de la fracción piomelanogénica en la totalidad de las poblaciones. Subsiguientemente, se procedió a la administración de ceftazidima, también durante tres días. En este caso, 17 de 27 poblaciones reprodujeron el ya aludido fenotipo de hiper-producción de piomelanina y, lo que es más trascendente, 23 de 27 exhibieron también hipersensibilidad a tobramicina. Es necesario reseñar que hasta 6 poblaciones desarrollaron la citada hipersensibilidad aun manteniendo *mexXY*; incógnita que ha de ser todavía investigada. Ahora bien, ante estas evidencias, y aun con sus limitaciones -como la ignota durabilidad de este fenotipo hipersensible-, es factible suponer que la sensibilidad colateral a tobramicina asociada al uso de ceftazidima podría representar una oportunidad de terapia explotable en la situación de poblaciones piomelanogénicas mixtas resistentes a ceftazidima de *P. aeruginosa* en FQ (coyuntura explorada en esta tesis) o en otras circunstancias menos complejas (poblaciones homogéneas o mixtas no resistentes a ceftazidima).

Antes de sacar partido a esa consabida hipersensibilidad a tobramicina de las poblaciones, se indagó en la posibilidad de que esta convergencia fenotípica se extendiera al rimero de resistencias cruzadas y sensibilidades colaterales a otros antimicrobianos de distintas familias de estas poblaciones. A lo largo de esta tesis se han detectado patrones pariguales en el fenotipo de sensibilidad a drogas dispares a la de selección en varias réplicas de *P. aeruginosa* PA14 sometidas a ALE, pero también existen ejemplos antitéticos en otros trabajos [329], por lo que se requería comprobación, tratándose además de diferentes fondos genéticos. En nuestro caso, todos ellos confluyeron hacia sensibilidad colateral a tetraciclina y fosfomicina. Esta última es un candidato asaz habitual en las propuestas de potenciales terapias combinadas para tratar infecciones debidas a *P. aeruginosa* [331-333]; es más, la combinación específica de tobramicina-fosfomicina es sinérgica frente a biopelículas de este patógeno en pacientes con FQ [271] y en ambientes anaerobios, al menos *in vitro* [272]. Consecuentemente, la pertinente administración de tobramicina para conducir a la extinción de las poblaciones hipersensibles resultantes del tratamiento alternado fue complementada con la alternativa del dúo tobramicina-fosfomicina, utilizando el doble de la CMI de cada uno de esos antimicrobianos. El resultado fue que 8 de 23 poblaciones resistieron a la tobramicina, y ninguna al tratamiento dual. En vista de ello, se propone que la combinación tobramicina-fosfomicina es la opción terapéutica más efectiva tras la alternancia de tobramicina y ceftazidima en la estrategia diseñada para estas condiciones y contextos genéticos de *P. aeruginosa* PA14. Eso sí, es menester recordar que su validez en el entorno hospitalario es dependiente de la conservación que posea el *trade-off* aquí analizado en aislados clínicos.

Al hilo de la fosfomicina, se ha de hacer un sucinto inciso para resaltar la llamativa hipersensibilidad a ésta que desarrollaron también todas las poblaciones de las ALEs pergeñadas en presencia de concentraciones letales de antibióticos, discutidas a lo largo de este trabajo. El trasfondo molecular de esta sensibilidad colateral aún está siendo investigado en el laboratorio, pero puede aseverarse que la conservación de este fenómeno apoya su posible candidatura para ser explotado en terapias combinadas o secuenciales con los antibióticos empleados en las citadas evoluciones experimentales, como se acaba de plantear para el caso concreto de la tobramicina.

En síntesis, con este postrer estudio, la discusión de la tesis se cierra rompiendo una lanza en favor del análisis de la convergencia fenotípica, en particular la concerniente a la sensibilidad colateral en mutantes resistentes de *P. aeruginosa*, puesto que desvela nuevos abordajes para tratar las infecciones debidas a este microorganismo, aun en circunstancias tan demandantes como las que plantean las poblaciones bacterianas mixtas en pacientes con FQ. Sirve como ejemplo perspicuo de la potencial aplicabilidad de los estudios predictivos de factores y efectos ligados a la evolución de la resistencia adquirida a los antibióticos en este patógeno oportunista. Estudios que, especialmente en el caso de los ensayos de ALE, han demostrado a lo largo de esta tesis ser muy eficaces para la predicción de dicha resistencia.

CONCLUSIONES

5. Conclusiones

I. El rol de un gen en el resistoma intrínseco de *P. aeruginosa* frente a un determinado aminoglicósido no es extrapolable al que desempeña frente a otros antibióticos de la misma familia estructural.

II. La pérdida de función de *glnD* propicia una disminución de la virulencia y la resistencia a diferentes antibióticos de *P. aeruginosa*, siendo por tanto este gen una diana prometedora para coadyuvantes de antibióticos que podrían emplearse en terapias anti-virulencia y anti-resistencia contra este patógeno.

III. Las trayectorias evolutivas hacia la resistencia frente a concentraciones letales de tobramicina, tigeciclina, ceftazidima y ceftazidima-avibactam en *P. aeruginosa* revisten cierto grado de conservación y, por tanto, predictibilidad. Esta convergencia es especialmente remarcable en el caso de la tobramicina.

IV. La evolución de *P. aeruginosa* en presencia de concentraciones letales de tigeciclina, a la que es intrínsecamente resistente, da lugar a un incremento en su nivel de resistencia a ésta, así como a la adquisición de resistencia cruzada a antibióticos clínicamente importantes, principalmente a causa de la sobre-expresión de *mexCD-oprJ*.

V. Existe una relación de epistasia negativa entre mutaciones de resistencia a antibióticos y mutaciones de pérdida de función en *lasR*, gen codificante de un regulador de QS en *P. aeruginosa*. Esto condiciona, genotípica y fenotípicamente, las rutas evolutivas hacia la resistencia a tobramicina y tigeciclina, y genera contingencia recíproca entre la virulencia y la resistencia a antibióticos de esta bacteria.

VI. Las trayectorias evolutivas de *P. aeruginosa* hacia la resistencia a drogas con diana en el ribosoma dependen de la presión de selección. Concentraciones subletales de tigeciclina pueden seleccionar resistencia a ésta y elevada resistencia cruzada a antimicrobianos de interés clínico, como las quinolonas. Sin embargo, concentraciones subletales de tobramicina (1/50 y 1/10 de la CMI de la cepa silvestre) son insuficientes para seleccionar mutantes resistentes.

VII. El primer paso hacia la adquisición de resistencia mutacional a ceftazidima y ceftazidima-avibactam en *P. aeruginosa* PA14 es la selección de grandes deleciones cromosómicas que ocasionan hiper-producción del pigmento piomelanina y sensibilidad

colateral a aminoglicósidos. Hasta un 13% de pacientes con FQ presentan cepas con estas características.

VIII. Se han identificado en *P. aeruginosa* mutaciones potencialmente involucradas en la resistencia a ceftazidima y su combinación con avibactam, siendo las que derivan en la sobre-expresión de *mexAB-oprM* las más prevalentes.

IX. Las rutas evolutivas tempranas hacia la resistencia a ceftazidima son robustas en mutantes de *P. aeruginosa* PA14 previamente resistentes a distintos antibióticos, y dan lugar a un fenotipo convergente de sensibilidad colateral a tobramicina.

X. El uso secuencial de tobramicina y ceftazidima, seguido de un regreso a la primera -o la combinación de ésta con fosfomicina-, se podría explotar para el tratamiento de infecciones crónicas causadas por poblaciones piomelanogénicas mixtas resistentes a ceftazidima de *P. aeruginosa*, dada la robustez del *trade-off* evolutivo de sensibilidad colateral a tobramicina.

BIBLIOGRAFÍA

6. Bibliografía

1 Davies, J. & Davies, D. Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* **74**, 417-433, doi:10.1128/MMBR.00016-10 (2010).

2 Abraham, E. P. & Chain, E. An enzyme from bacteria able to destroy penicillin. 1940. *Rev Infect Dis* **10**, 677-678 (1988).

3 CDC. *About Antibiotic Resistance*. <https://www.cdc.gov/drugresistance/about.html>.

4 da Costa, P. M., Loureiro, L. & Matos, A. J. Transfer of multidrug-resistant bacteria between intermingled ecological niches: the interface between humans, animals and the environment. *Int J Environ Res Public Health* **10**, 278-294, doi:10.3390/ijerph10010278 (2013).

5 Manyi-Loh, C., Mamphweli, S., Meyer, E. & Okoh, A. Antibiotic Use in Agriculture and Its Consequential Resistance in Environmental Sources: Potential Public Health Implications. *Molecules* **23**, doi:10.3390/molecules23040795 (2018).

6 Hernando-Amado, S., Coque, T. M., Baquero, F. & Martinez, J. L. Defining and combating antibiotic resistance from One Health and Global Health perspectives. *Nat Microbiol* **4**, 1432-1442, doi:10.1038/s41564-019-0503-9 (2019).

7 Ventola, C. L. The antibiotic resistance crisis: part 1: causes and threats. *P T* **40**, 277-283 (2015).

8 Viswanathan, V. K. Off-label abuse of antibiotics by bacteria. *Gut Microbes* **5**, 3-4, doi:10.4161/gmic.28027 (2014).

9 Gross, M. Antibiotics in crisis. *Curr Biol* **23**, R1063-1065, doi:10.1016/j.cub.2013.11.057 (2013).

10 Hendriksen, R. S. *et al.* Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nat Commun* **10**, 1124, doi:10.1038/s41467-019-08853-3 (2019).

11 Van Valen, L. A New Evolutionary Law. *Evol. Theory* **1**, 1-30 (1973).

12 Carroll, L. Through the Looking-Glass. (1871).

13 Cassini, A. *et al.* Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *Lancet Infect Dis* **19**, 56-66, doi:10.1016/S1473-3099(18)30605-4 (2019).

14 WHO. More People in the United States Dying from Antibiotic-Resistant Infections than Previously Estimated. (2019).

15 WHO. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. (2017).

16 Santajit, S. & Indrawattana, N. Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. *Biomed Res Int* **2016**, 2475067, doi:10.1155/2016/2475067 (2016).

17 Rello, J. *et al.* A global priority list of the TOp TEn resistant Microorganisms (TOTEM) study at intensive care: a prioritization exercise based on multi-criteria decision analysis. *Eur J Clin Microbiol Infect Dis* **38**, 319-323, doi:10.1007/s10096-018-3428-y (2019).

18 Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O. & Piddock, L. J. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* **13**, 42-51, doi:10.1038/nrmicro3380 (2015).

19 Turnidge, J., Kahlmeter, G. & Kronvall, G. Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. *Clin Microbiol Infect* **12**, 418-425, doi:10.1111/j.1469-0691.2006.01377.x (2006).

20 Fajardo, A. *et al.* The neglected intrinsic resistome of bacterial pathogens. *PLoS One* **3**, e1619, doi:10.1371/journal.pone.0001619 (2008).

21 Olivares, J. *et al.* The intrinsic resistome of bacterial pathogens. *Front Microbiol* **4**, 103, doi:10.3389/fmicb.2013.00103 (2013).

22 Alonso, A., Sanchez, P. & Martinez, J. L. Environmental selection of antibiotic resistance genes. *Environ Microbiol* **3**, 1-9, doi:10.1046/j.1462-2920.2001.00161.x (2001).

23 Liu, A. *et al.* Antibiotic sensitivity profiles determined with an Escherichia coli gene knockout collection: generating an antibiotic bar code. *Antimicrob Agents Chemother* **54**, 1393-1403, doi:10.1128/AAC.00906-09 (2010).

24 Bernardini, A. *et al.* The intrinsic resistome of Klebsiella pneumoniae. *Int J Antimicrob Agents* **53**, 29-33, doi:10.1016/j.ijantimicag.2018.09.012 (2019).

25 Schurek, K. N. *et al.* Novel genetic determinants of low-level aminoglycoside resistance in Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **52**, 4213-4219, doi:10.1128/AAC.00507-08 (2008).

26 Tamae, C. *et al.* Determination of antibiotic hypersensitivity among 4,000 single-gene-knockout mutants of Escherichia coli. *J Bacteriol* **190**, 5981-5988, doi:10.1128/JB.01982-07 (2008).

27 Nikaido, H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **264**, 382-388, doi:10.1126/science.8153625 (1994).

28 Randall, C. P., Mariner, K. R., Chopra, I. & O'Neill, A. J. The target of daptomycin is absent from Escherichia coli and other gram-negative pathogens. *Antimicrob Agents Chemother* **57**, 637-639, doi:10.1128/AAC.02005-12 (2013).

29 Zhu, L., Lin, J., Ma, J., Cronan, J. E. & Wang, H. Triclosan resistance of Pseudomonas aeruginosa PAO1 is due to FabV, a triclosan-resistant enoyl-acyl carrier protein reductase. *Antimicrob Agents Chemother* **54**, 689-698, doi:10.1128/AAC.01152-09 (2010).

30 Egorov, A. M., Ulyashova, M. M. & Rubtsova, M. Y. Bacterial Enzymes and Antibiotic Resistance. *Acta Naturae* **10**, 33-48 (2018).

31 Bush, K. & Jacoby, G. A. Updated functional classification of beta-lactamases. *Antimicrob Agents Chemother* **54**, 969-976, doi:10.1128/AAC.01009-09 (2010).

32 Morar, M., Pengelly, K., Koteva, K. & Wright, G. D. Mechanism and diversity of the erythromycin esterase family of enzymes. *Biochemistry* **51**, 1740-1751, doi:10.1021/bi201790u (2012).

33 Jacoby, G. A. AmpC beta-lactamases. *Clin Microbiol Rev* **22**, 161-182, Table of Contents, doi:10.1128/CMR.00036-08 (2009).

34 Wright, G. D. Bacterial resistance to antibiotics: enzymatic degradation and modification. *Adv Drug Deliv Rev* **57**, 1451-1470, doi:10.1016/j.addr.2005.04.002 (2005).

35 Tsuchido, T. & Takano, M. Sensitization by heat treatment of Escherichia coli K-12 cells to hydrophobic antibacterial compounds. *Antimicrob Agents Chemother* **32**, 1680-1683, doi:10.1128/aac.32.11.1680 (1988).

36 Delcour, A. H. Outer membrane permeability and antibiotic resistance. *Biochim Biophys Acta* **1794**, 808-816, doi:10.1016/j.bbapap.2008.11.005 (2009).

37 Zgurskaya, H. I., Lopez, C. A. & Gnanakaran, S. Permeability Barrier of Gram-Negative Cell Envelopes and Approaches To Bypass It. *ACS Infect Dis* **1**, 512-522, doi:10.1021/acsinfecdis.5b00097 (2015).

38 Du, D. *et al.* Multidrug efflux pumps: structure, function and regulation. *Nat Rev Microbiol* **16**, 523-539, doi:10.1038/s41579-018-0048-6 (2018).

39 Daury, L. *et al.* Tripartite assembly of RND multidrug efflux pumps. *Nat Commun* **7**, 10731, doi:10.1038/ncomms10731 (2016).

40 Murakami, S., Nakashima, R., Yamashita, E. & Yamaguchi, A. Crystal structure of bacterial multidrug efflux transporter AcrB. *Nature* **419**, 587-593, doi:10.1038/nature01050 (2002).

41 Su, C. C. *et al.* Structures and transport dynamics of a Campylobacter jejuni multidrug efflux pump. *Nat Commun* **8**, 171, doi:10.1038/s41467-017-00217-z (2017).

42 Li, X. Z., Nikaido, H. & Poole, K. Role of mexA-mexB-oprM in antibiotic efflux in Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **39**, 1948-1953, doi:10.1128/aac.39.9.1948 (1995).

43 Rosenberg, E. Y., Bertenthal, D., Nilles, M. L., Bertrand, K. P. & Nikaido, H. Bile salts and fatty acids induce the expression of Escherichia coli AcrAB multidrug efflux pump through their interaction with Rob regulatory protein. *Mol Microbiol* **48**, 1609-1619, doi:10.1046/j.1365-2958.2003.03531.x (2003).

44 Krahn, T. *et al.* Determinants of intrinsic aminoglycoside resistance in Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **56**, 5591-5602, doi:10.1128/AAC.01446-12 (2012).

45 Linares, J. F. *et al.* The global regulator Crc modulates metabolism, susceptibility to antibiotics and virulence in Pseudomonas aeruginosa. *Environ Microbiol* **12**, 3196-3212, doi:10.1111/j.1462-2920.2010.02292.x (2010).

46 Martinez, J. L. & Rojo, F. Metabolic regulation of antibiotic resistance. *FEMS Microbiol Rev* **35**, 768-789, doi:10.1111/j.1574-6976.2011.00282.x (2011).

47 Sougakoff, W., Goussard, S., Gerbaud, G. & Courvalin, P. Plasmid-mediated resistance to third-generation cephalosporins caused by point mutations in TEM-type penicillinase genes. *Rev Infect Dis* **10**, 879-884, doi:10.1093/clinids/10.4.879 (1988).

48 Woodford, N. & Ellington, M. J. The emergence of antibiotic resistance by mutation. *Clin Microbiol Infect* **13**, 5-18, doi:10.1111/j.1469-0691.2006.01492.x (2007).

49 Gillespie, S. H. Evolution of drug resistance in Mycobacterium tuberculosis: clinical and molecular perspective. *Antimicrob Agents Chemother* **46**, 267-274, doi:10.1128/aac.46.2.267-274.2002 (2002).

50 Hu, M., Nandi, S., Davies, C. & Nicholas, R. A. High-level chromosomally mediated tetracycline resistance in Neisseria gonorrhoeae results from a point mutation in the rpsJ gene encoding ribosomal protein S10 in combination with the mtrR and penB resistance determinants. *Antimicrob Agents Chemother* **49**, 4327-4334, doi:10.1128/AAC.49.10.4327-4334.2005 (2005).

51 Ahn, C., Yoon, S. S., Yong, T. S., Jeong, S. H. & Lee, K. The Resistance Mechanism and Clonal Distribution of Tigecycline-Nonsusceptible Klebsiella pneumoniae Isolates in Korea. *Yonsei Med J* **57**, 641-646, doi:10.3349/ymj.2016.57.3.641 (2016).

52 Poole, K. *et al.* Expression of the multidrug resistance operon mexA-mexB-oprM in Pseudomonas aeruginosa: mexR encodes a regulator of operon expression. *Antimicrob Agents Chemother* **40**, 2021-2028 (1996).

53 Lin, J., Akiba, M., Sahin, O. & Zhang, Q. CmeR functions as a transcriptional repressor for the multidrug efflux pump CmeABC in Campylobacter jejuni. *Antimicrob Agents Chemother* **49**, 1067-1075, doi:10.1128/AAC.49.3.1067-1075.2005 (2005).

54 Veal, W. L., Nicholas, R. A. & Shafer, W. M. Overexpression of the MtrC-MtrD-MtrE efflux pump due to an mtrR mutation is required for chromosomally mediated penicillin resistance in Neisseria gonorrhoeae. *J Bacteriol* **184**, 5619-5624, doi:10.1128/jb.184.20.5619-5624.2002 (2002).

55 Blanco, P., Corona, F. & Martinez, J. L. Involvement of the RND efflux pump transporter SmeH in the acquisition of resistance to ceftazidime in Stenotrophomonas maltophilia. *Sci Rep* **9**, 4917, doi:10.1038/s41598-019-41308-9 (2019).

56 Vettoretti, L. *et al.* Efflux unbalance in Pseudomonas aeruginosa isolates from cystic fibrosis patients. *Antimicrob Agents Chemother* **53**, 1987-1997, doi:10.1128/AAC.01024-08 (2009).

57 Blair, J. M. *et al.* AcrB drug-binding pocket substitution confers clinically relevant resistance and altered substrate specificity. *Proc Natl Acad Sci U S A* **112**, 3511-3516, doi:10.1073/pnas.1419939112 (2015).

58 Hanson, N. D. AmpC beta-lactamases: what do we need to know for the future? *J Antimicrob Chemother* **52**, 2-4, doi:10.1093/jac/dkg284 (2003).

59 Hanson, N. D. & Sanders, C. C. Regulation of inducible AmpC beta-lactamase expression among Enterobacteriaceae. *Curr Pharm Des* **5**, 881-894 (1999).

60 Morosini, M. I. *et al.* An extended-spectrum AmpC-type beta-lactamase obtained by in vitro antibiotic selection. *FEMS Microbiol Lett* **165**, 85-90, doi:10.1111/j.1574-6968.1998.tb13131.x (1998).

61 Vakulenko, S. B. *et al.* Mutational replacement of Leu-293 in the class C Enterobacter cloacae P99 beta-lactamase confers increased MIC of cefepime. *Antimicrob Agents Chemother* **46**, 1966-1970, doi:10.1128/aac.46.6.1966-1970.2002 (2002).

62 Barnaud, G. *et al.* Selection during cefepime treatment of a new cephalosporinase variant with extended-spectrum resistance to cefepime in an Enterobacter aerogenes clinical isolate. *Antimicrob Agents Chemother* **48**, 1040-1042, doi:10.1128/aac.48.3.1040-1042.2004 (2004).

63 Mammeri, H., Poirel, L., Bemer, P., Drugeon, H. & Nordmann, P. Resistance to cefepime and cefpirome due to a 4-amino-acid deletion in the chromosome-encoded AmpC beta-lactamase of a Serratia marcescens clinical isolate. *Antimicrob Agents Chemother* **48**, 716-720, doi:10.1128/aac.48.3.716-720.2004 (2004).

64 Fernandez, L. & Hancock, R. E. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clin Microbiol Rev* **25**, 661-681, doi:10.1128/CMR.00043-12 (2012).

65 Knopp, M. & Andersson, D. I. Amelioration of the Fitness Costs of Antibiotic Resistance Due To Reduced Outer Membrane Permeability by Upregulation of Alternative Porins. *Mol Biol Evol* **32**, 3252-3263, doi:10.1093/molbev/msv195 (2015).

66 Thiolas, A., Bornet, C., Davin-Regli, A., Pages, J. M. & Bollet, C. Resistance to imipenem, cefepime, and cefpirome associated with mutation in Omp36 osmoporin of Enterobacter aerogenes. *Biochem Biophys Res Commun* **317**, 851-856, doi:10.1016/j.bbrc.2004.03.130 (2004).

67 Hiramatsu, K. Vancomycin-resistant Staphylococcus aureus: a new model of antibiotic resistance. *Lancet Infect Dis* **1**, 147-155, doi:10.1016/S1473-3099(01)00091-3 (2001).

68 Zhao, S., Tobiason, D. M., Hu, M., Seifert, H. S. & Nicholas, R. A. The penC mutation conferring antibiotic resistance in Neisseria gonorrhoeae arises from a mutation in the PilQ secretin that interferes with multimer stability. *Mol Microbiol* **57**, 1238-1251, doi:10.1111/j.1365-2958.2005.04752.x (2005).

69 Lopez-Causape, C., Rubio, R., Cabot, G. & Oliver, A. Evolution of the Pseudomonas aeruginosa Aminoglycoside Mutational Resistome In Vitro and in the Cystic Fibrosis Setting. *Antimicrob Agents Chemother* **62**, doi:10.1128/AAC.02583-17 (2018).

70 Larranaga, O. *et al.* Phage particles harboring antibiotic resistance genes in fresh-cut vegetables and agricultural soil. *Environ Int* **115**, 133-141, doi:10.1016/j.envint.2018.03.019 (2018).

71 Bennett, P. M. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br J Pharmacol* **153 Suppl 1**, S347-357, doi:10.1038/sj.bjp.0707607 (2008).

72 Babakhani, S. & Oloomi, M. Transposons: the agents of antibiotic resistance in bacteria. *J Basic Microbiol* **58**, 905-917, doi:10.1002/jobm.201800204 (2018).

73 Paterson, D. L. & Bonomo, R. A. Extended-spectrum beta-lactamases: a clinical update. *Clin Microbiol Rev* **18**, 657-686, doi:10.1128/CMR.18.4.657-686.2005 (2005).

74 Mulvey, M. R., Boyd, D. A., Olson, A. B., Doublet, B. & Cloeckaert, A. The genetics of Salmonella genomic island 1. *Microbes Infect* **8**, 1915-1922, doi:10.1016/j.micinf.2005.12.028 (2006).

75 Corona, F. & Martinez, J. L. Phenotypic Resistance to Antibiotics. *Antibiotics (Basel)* **2**, 237-255, doi:10.3390/antibiotics2020237 (2013).

76 Mc, D. W. Microbial persistence. *Yale J Biol Med* **30**, 257-291 (1958).

77 Costerton, J. W., Stewart, P. S. & Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318-1322, doi:10.1126/science.284.5418.1318 (1999).

78 Kubicek-Sutherland, J. Z. *et al.* Host-dependent Induction of Transient Antibiotic Resistance: A Prelude to Treatment Failure. *EBioMedicine* **2**, 1169-1178, doi:10.1016/j.ebiom.2015.08.012 (2015).

79 Fernandez, L., Breidenstein, E. B. & Hancock, R. E. Creeping baselines and adaptive resistance to antibiotics. *Drug Resist Updat* **14**, 1-21, doi:10.1016/j.drup.2011.01.001 (2011).

80 Lin, J. *et al.* Bile salts modulate expression of the CmeABC multidrug efflux pump in Campylobacter jejuni. *J Bacteriol* **187**, 7417-7424, doi:10.1128/JB.187.21.7417-7424.2005 (2005).

81 Blanco, P., Corona, F. & Martinez, J. L. Biolog Phenotype Microarray Is a Tool for the Identification of Multidrug Resistance Efflux Pump Inducers. *Antimicrob Agents Chemother* **62**, doi:10.1128/AAC.01263-18 (2018).

82 Hall, C. W. & Mah, T. F. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiol Rev* **41**, 276-301, doi:10.1093/femsre/fux010 (2017).

83 Laborda, P., Alcalde-Rico, M., Blanco, P., Martinez, J. L. & Hernando-Amado, S. Novel Inducers of the Expression of Multidrug Efflux Pumps That Trigger Pseudomonas aeruginosa Transient Antibiotic Resistance. *Antimicrob Agents Chemother* **63**, doi:10.1128/AAC.01095-19 (2019).

84 Garcia-Leon, G. *et al.* A function of SmeDEF, the major quinolone resistance determinant of Stenotrophomonas maltophilia, is the colonization of plant roots. *Appl Environ Microbiol* **80**, 4559-4565, doi:10.1128/AEM.01058-14 (2014).

85 Hernandez, A., Ruiz, F. M., Romero, A. & Martinez, J. L. The binding of triclosan to SmeT, the repressor of the multidrug efflux pump SmeDEF, induces antibiotic resistance in Stenotrophomonas maltophilia. *PLoS Pathog* **7**, e1002103, doi:10.1371/journal.ppat.1002103 (2011).

86 Blanco, P., Corona, F., Sanchez, M. B. & Martinez, J. L. Vitamin K3 Induces the Expression of the Stenotrophomonas maltophilia SmeVWX Multidrug Efflux Pump. *Antimicrob Agents Chemother* **61**, doi:10.1128/AAC.02453-16 (2017).

87 Macfarlane, E. L., Kwasnicka, A., Ochs, M. M. & Hancock, R. E. PhoP-PhoQ homologues in Pseudomonas aeruginosa regulate expression of the outer-membrane protein OprH and polymyxin B resistance. *Mol Microbiol* **34**, 305-316, doi:10.1046/j.1365-2958.1999.01600.x (1999).

88 McPhee, J. B., Lewenza, S. & Hancock, R. E. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in Pseudomonas aeruginosa. *Mol Microbiol* **50**, 205-217, doi:10.1046/j.1365-2958.2003.03673.x (2003).

89 Kox, L. F., Wosten, M. M. & Groisman, E. A. A small protein that mediates the activation of a two-component system by another two-component system. *EMBO J* **19**, 1861-1872, doi:10.1093/emboj/19.8.1861 (2000).

90 Mortimer, P. G. & Piddock, L. J. The accumulation of five antibacterial agents in porin-deficient mutants of Escherichia coli. *J Antimicrob Chemother* **32**, 195-213, doi:10.1093/jac/32.2.195 (1993).

91 Dupont, M., James, C. E., Chevalier, J. & Pages, J. M. An early response to environmental stress involves regulation of OmpX and OmpF, two enterobacterial outer membrane pore-forming proteins. *Antimicrob Agents Chemother* **51**, 3190-3198, doi:10.1128/AAC.01481-06 (2007).

92 Lazar, V. & Kishony, R. Transient antibiotic resistance calls for attention. *Nat Microbiol* **4**, 1606-1607, doi:10.1038/s41564-019-0571-x (2019).

93 Andersson, D. I., Nicoloff, H. & Hjort, K. Mechanisms and clinical relevance of bacterial heteroresistance. *Nat Rev Microbiol* **17**, 479-496, doi:10.1038/s41579-019-0218-1 (2019).

94 Andersson, D. I. & Levin, B. R. The biological cost of antibiotic resistance. *Curr Opin Microbiol* **2**, 489-493, doi:10.1016/s1369-5274(99)00005-3 (1999).

95 Bjorkman, J. & Andersson, D. I. The cost of antibiotic resistance from a bacterial perspective. *Drug Resist Updat* **3**, 237-245, doi:10.1054/drup.2000.0147 (2000).

96 Andersson, D. I. & Hughes, D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol* **8**, 260-271, doi:10.1038/nrmicro2319 (2010).

97 Choi, M. J. & Ko, K. S. Loss of hypermucoviscosity and increased fitness cost in colistin-resistant Klebsiella pneumoniae sequence type 23 strains. *Antimicrob Agents Chemother* **59**, 6763-6773, doi:10.1128/AAC.00952-15 (2015).

98 Cohen, T., Sommers, B. & Murray, M. The effect of drug resistance on the fitness of Mycobacterium tuberculosis. *Lancet Infect Dis* **3**, 13-21, doi:10.1016/s1473-3099(03)00483-3 (2003).

99 Baquero, F., Alvarez-Ortega, C. & Martinez, J. L. Ecology and evolution of antibiotic resistance. *Environ Microbiol Rep* **1**, 469-476, doi:10.1111/j.1758-2229.2009.00053.x (2009).

100 Tian, Z. X. *et al.* Transcriptome profiling defines a novel regulon modulated by the LysR-type transcriptional regulator MexT in Pseudomonas aeruginosa. *Nucleic Acids Res* **37**, 7546-7559, doi:10.1093/nar/gkp828 (2009).

101 Balasubramanian, D. *et al.* The regulatory repertoire of Pseudomonas aeruginosa AmpC ss-lactamase regulator AmpR includes virulence genes. *PLoS One* **7**, e34067, doi:10.1371/journal.pone.0034067 (2012).

102 John, J. F., Jr. & Rice, L. B. The microbial genetics of antibiotic cycling. *Infect Control Hosp Epidemiol* **21**, S22-31, doi:10.1086/503170 (2000).

103 van Loon, H. J. *et al.* Antibiotic rotation and development of gram-negative antibiotic resistance. *Am J Respir Crit Care Med* **171**, 480-487, doi:10.1164/rccm.200401-070OC (2005).

104 Luo, N. *et al.* Enhanced in vivo fitness of fluoroquinolone-resistant Campylobacter jejuni in the absence of antibiotic selection pressure. *Proc Natl Acad Sci U S A* **102**, 541-546, doi:10.1073/pnas.0408966102 (2005).

105 Fuzi, M. Dissimilar Fitness Associated with Resistance to Fluoroquinolones Influences Clonal Dynamics of Various Multiresistant Bacteria. *Front Microbiol* **7**, 1017, doi:10.3389/fmicb.2016.01017 (2016).

106 Dench, J., Hinz, A., Aris-Brosou, S. & Kassen, R. Identifying the drivers of computationally detected correlated evolution among sites under antibiotic selection. *Evol Appl* **13**, 781-793, doi:10.1111/eva.12900 (2020).

107 Sherman, D. R. *et al.* Compensatory ahpC gene expression in isoniazid-resistant Mycobacterium tuberculosis. *Science* **272**, 1641-1643, doi:10.1126/science.272.5268.1641 (1996).

108 Marcusson, L. L., Frimodt-Moller, N. & Hughes, D. Interplay in the selection of fluoroquinolone resistance and bacterial fitness. *PLoS Pathog* **5**, e1000541, doi:10.1371/journal.ppat.1000541 (2009).

109 Trindade, S. *et al.* Positive epistasis drives the acquisition of multidrug resistance. *PLoS Genet* **5**, e1000578, doi:10.1371/journal.pgen.1000578 (2009).

110 Hall, A. R. & MacLean, R. C. Epistasis buffers the fitness effects of rifampicin- resistance mutations in Pseudomonas aeruginosa. *Evolution* **65**, 2370-2379, doi:10.1111/j.1558-5646.2011.01302.x (2011).

111 Durao, P., Trindade, S., Sousa, A. & Gordo, I. Multiple Resistance at No Cost: Rifampicin and Streptomycin a Dangerous Liaison in the Spread of Antibiotic Resistance. *Mol Biol Evol* **32**, 2675-2680, doi:10.1093/molbev/msv143 (2015).

112 Nilsson, A. I. *et al.* Reducing the fitness cost of antibiotic resistance by amplification of initiator tRNA genes. *Proc Natl Acad Sci U S A* **103**, 6976-6981, doi:10.1073/pnas.0602171103 (2006).

113 Olivares Pacheco, J., Alvarez-Ortega, C., Alcalde Rico, M. & Martinez, J. L. Metabolic Compensation of Fitness Costs Is a General Outcome for Antibiotic-Resistant Pseudomonas aeruginosa Mutants Overexpressing Efflux Pumps. *mBio* **8**, doi:10.1128/mBio.00500-17 (2017).

114 Freihofer, P. *et al.* Nonmutational compensation of the fitness cost of antibiotic resistance in mycobacteria by overexpression of tlyA rRNA methylase. *RNA* **22**, 1836-1843, doi:10.1261/rna.057257.116 (2016).

115 Hernando-Amado, S., Sanz-Garcia, F., Blanco, P. & Martinez, J. L. Fitness costs associated with the acquisition of antibiotic resistance. *Essays Biochem* **61**, 37-48, doi:10.1042/EBC20160057 (2017).

116 Szybalski, W. & Bryson, V. Genetic studies on microbial cross resistance to toxic agents. I. Cross resistance of Escherichia coli to fifteen antibiotics. *J Bacteriol* **64**, 489-499 (1952).

117 Webber, M. A. & Piddock, L. J. The importance of efflux pumps in bacterial antibiotic resistance. *J Antimicrob Chemother* **51**, 9-11, doi:10.1093/jac/dkg050 (2003).

118 Sanders, C. C. Mechanisms responsible for cross-resistance and dichotomous resistance among the quinolones. *Clin Infect Dis* **32 Suppl 1**, S1-8, doi:10.1086/319369 (2001).

119 Oz, T. *et al.* Strength of selection pressure is an important parameter contributing to the complexity of antibiotic resistance evolution. *Mol Biol Evol* **31**, 2387-2401, doi:10.1093/molbev/msu191 (2014).

120 Webber, M. A. *et al.* Clinically relevant mutant DNA gyrase alters supercoiling, changes the transcriptome, and confers multidrug resistance. *mBio* **4**, doi:10.1128/mBio.00273-13 (2013).

121 Lazar, V. *et al.* Genome-wide analysis captures the determinants of the antibiotic cross-resistance interaction network. *Nat Commun* **5**, 4352, doi:10.1038/ncomms5352 (2014).

122 Roemhild, R., Linkevicius, M. & Andersson, D. I. Molecular mechanisms of collateral sensitivity to the antibiotic nitrofurantoin. *PLoS Biol* **18**, e3000612, doi:10.1371/journal.pbio.3000612 (2020).

123 Imamovic, L. & Sommer, M. O. Use of collateral sensitivity networks to design drug cycling protocols that avoid resistance development. *Sci Transl Med* **5**, 204ra132, doi:10.1126/scitranslmed.3006609 (2013).

124 Nichol, D. *et al.* Antibiotic collateral sensitivity is contingent on the repeatability of evolution. *Nat Commun* **10**, 334, doi:10.1038/s41467-018-08098-6 (2019).

125 Rosenkilde, C. E. H. *et al.* Collateral sensitivity constrains resistance evolution of the CTX-M-15 beta-lactamase. *Nat Commun* **10**, 618, doi:10.1038/s41467-019-08529-y (2019).

126 Lazar, V. *et al.* Bacterial evolution of antibiotic hypersensitivity. *Mol Syst Biol* **9**, 700, doi:10.1038/msb.2013.57 (2013).

127 Pal, C., Papp, B. & Lazar, V. Collateral sensitivity of antibiotic-resistant microbes. *Trends Microbiol* **23**, 401-407, doi:10.1016/j.tim.2015.02.009 (2015).

128 Munck, C., Gumpert, H. K., Wallin, A. I., Wang, H. H. & Sommer, M. O. Prediction of resistance development against drug combinations by collateral responses to component drugs. *Sci Transl Med* **6**, 262ra156, doi:10.1126/scitranslmed.3009940 (2014).

129 Barbosa, C., Beardmore, R., Schulenburg, H. & Jansen, G. Antibiotic combination efficacy (ACE) networks for a Pseudomonas aeruginosa model. *PLoS Biol* **16**, e2004356, doi:10.1371/journal.pbio.2004356 (2018).

130 Kim, S., Lieberman, T. D. & Kishony, R. Alternating antibiotic treatments constrain evolutionary paths to multidrug resistance. *Proc Natl Acad Sci U S A* **111**, 14494-14499, doi:10.1073/pnas.1409800111 (2014).

131 Roemhild, R. & Schulenburg, H. Evolutionary ecology meets the antibiotic crisis: Can we control pathogen adaptation through sequential therapy? *Evol Med Public Health* **2019**, 37-45, doi:10.1093/emph/eoz008 (2019).

132 Barbosa, C., Romhild, R., Rosenstiel, P. & Schulenburg, H. Evolutionary stability of collateral sensitivity to antibiotics in the model pathogen Pseudomonas aeruginosa. *Elife* **8**, doi:10.7554/eLife.51481 (2019).

133 Knopp, M. & Andersson, D. I. Predictable Phenotypes of Antibiotic Resistance Mutations. *mBio* **9**, doi:10.1128/mBio.00770-18 (2018).

134 Podnecky, N. L. *et al.* Conserved collateral antibiotic susceptibility networks in diverse clinical strains of Escherichia coli. *Nat Commun* **9**, 3673, doi:10.1038/s41467-018-06143-y (2018).

135 Apjok, G. *et al.* Limited Evolutionary Conservation of the Phenotypic Effects of Antibiotic Resistance Mutations. *Mol Biol Evol* **36**, 1601-1611, doi:10.1093/molbev/msz109 (2019).

136 Lenormand, T., Roze, D. & Rousset, F. Stochasticity in evolution. *Trends Ecol Evol* **24**, 157-165, doi:10.1016/j.tree.2008.09.014 (2009).

137 Martinez, J. L., Baquero, F. & Andersson, D. I. Predicting antibiotic resistance. *Nat Rev Microbiol* **5**, 958-965, doi:10.1038/nrmicro1796 (2007).

138 Hughes, D. & Andersson, D. I. Evolutionary Trajectories to Antibiotic Resistance. *Annu Rev Microbiol* **71**, 579-596, doi:10.1146/annurev-micro-090816-093813 (2017).

139 Her, H. L. & Wu, Y. W. A pan-genome-based machine learning approach for predicting antimicrobial resistance activities of the Escherichia coli strains. *Bioinformatics* **34**, i89-i95, doi:10.1093/bioinformatics/bty276 (2018).

140 Moradigaravand, D. *et al.* Prediction of antibiotic resistance in Escherichia coli from large-scale pan-genome data. *PLoS Comput Biol* **14**, e1006258, doi:10.1371/journal.pcbi.1006258 (2018).

141 Khaledi, A. *et al.* Predicting antimicrobial resistance in Pseudomonas aeruginosa with machine learning-enabled molecular diagnostics. *EMBO Mol Med* **12**, e10264, doi:10.15252/emmm.201910264 (2020).

142 Lopez-Causape, C. *et al.* Evolution of the Pseudomonas aeruginosa mutational resistome in an international Cystic Fibrosis clone. *Sci Rep* **7**, 5555, doi:10.1038/s41598-017-05621-5 (2017).

143 Macia, M. D. *et al.* Efficacy and potential for resistance selection of antipseudomonal treatments in a mouse model of lung infection by hypermutable Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **50**, 975-983, doi:10.1128/AAC.50.3.975-983.2006 (2006).

144 Alvarez-Ortega, C., Wiegand, I., Olivares, J., Hancock, R. E. & Martinez, J. L. The intrinsic resistome of Pseudomonas aeruginosa to beta-lactams. *Virulence* **2**, 144-146, doi:10.4161/viru.2.2.15014 (2011).

145 Fernandez, L. *et al.* Characterization of the polymyxin B resistome of Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **57**, 110-119, doi:10.1128/AAC.01583-12 (2013).

146 Breidenstein, E. B., Khaira, B. K., Wiegand, I., Overhage, J. & Hancock, R. E. Complex ciprofloxacin resistome revealed by screening a Pseudomonas aeruginosa mutant library for altered susceptibility. *Antimicrob Agents Chemother* **52**, 4486-4491, doi:10.1128/AAC.00222-08 (2008).

147 Blake, K. L. & O'Neill, A. J. Transposon library screening for identification of genetic loci participating in intrinsic susceptibility and acquired resistance to antistaphylococcal agents. *J Antimicrob Chemother* **68**, 12-16, doi:10.1093/jac/dks373 (2013).

148 Vestergaard, M. *et al.* Genome-Wide Identification of Antimicrobial Intrinsic Resistance Determinants in Staphylococcus aureus. *Front Microbiol* **7**, 2018, doi:10.3389/fmicb.2016.02018 (2016).

149 Gomez, M. J. & Neyfakh, A. A. Genes involved in intrinsic antibiotic resistance of Acinetobacter baylyi. *Antimicrob Agents Chemother* **50**, 3562-3567, doi:10.1128/AAC.00579-06 (2006).

150 Shin, S. J., Wu, C. W., Steinberg, H. & Talaat, A. M. Identification of novel virulence determinants in Mycobacterium paratuberculosis by screening a library of insertional mutants. *Infect Immun* **74**, 3825-3833, doi:10.1128/IAI.01742-05 (2006).

151 Guo, Q. *et al.* Identification of a small molecule that simultaneously suppresses virulence and antibiotic resistance of Pseudomonas aeruginosa. *Sci Rep* **6**, 19141, doi:10.1038/srep19141 (2016).

152 Bouma, J. E. & Lenski, R. E. Evolution of a bacteria/plasmid association. *Nature* **335**, 351-352, doi:10.1038/335351a0 (1988).

153 Bryson, V. & Szybalski, W. Microbial Selection. *Science* **116**, 45-51, doi:10.1126/science.116.3003.45 (1952).

154 Toprak, E. *et al.* Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nat Genet* **44**, 101-105, doi:10.1038/ng.1034 (2011).

155 Feng, Y., Jonker, M. J., Moustakas, I., Brul, S. & Ter Kuile, B. H. Dynamics of Mutations during Development of Resistance by Pseudomonas aeruginosa against Five Antibiotics. *Antimicrob Agents Chemother* **60**, 4229-4236, doi:10.1128/AAC.00434-16 (2016).

156 Ibacache-Quiroga, C., Oliveros, J. C., Couce, A. & Blazquez, J. Parallel Evolution of High-Level Aminoglycoside Resistance in Escherichia coli Under Low and High Mutation Supply Rates. *Front Microbiol* **9**, 427, doi:10.3389/fmicb.2018.00427 (2018).

157 Martinez, J. L., Baquero, F. & Andersson, D. I. Beyond serial passages: new methods for predicting the emergence of resistance to novel antibiotics. *Curr Opin Pharmacol* **11**, 439-445, doi:10.1016/j.coph.2011.07.005 (2011).

158 Wardell, S. J. T. *et al.* A large-scale whole-genome comparison shows that experimental evolution in response to antibiotics predicts changes in naturally evolved clinical Pseudomonas aeruginosa. *Antimicrob Agents Chemother*, doi:10.1128/AAC.01619-19 (2019).

159 Jochumsen, N. *et al.* The evolution of antimicrobial peptide resistance in Pseudomonas aeruginosa is shaped by strong epistatic interactions. *Nat Commun* **7**, 13002, doi:10.1038/ncomms13002 (2016).

160 Cabot, G. *et al.* Evolution of Pseudomonas aeruginosa Antimicrobial Resistance and Fitness under Low and High Mutation Rates. *Antimicrob Agents Chemother* **60**, 1767-1778, doi:10.1128/AAC.02676-15 (2016).

161 Blanco, P., Corona, F. & Martinez, J. L. Mechanisms and phenotypic consequences of acquisition of tigecycline resistance by Stenotrophomonas maltophilia. *J Antimicrob Chemother* **74**, 3221-3230, doi:10.1093/jac/dkz326 (2019).

162 Shcherbakov, D. *et al.* Directed mutagenesis of Mycobacterium smegmatis 16S rRNA to reconstruct the in vivo evolution of aminoglycoside resistance in Mycobacterium tuberculosis. *Mol Microbiol* **77**, 830-840, doi:10.1111/j.1365-2958.2010.07218.x (2010).

163 Weinreich, D. M. The rank ordering of genotypic fitness values predicts genetic constraint on natural selection on landscapes lacking sign epistasis. *Genetics* **171**, 1397-1405, doi:10.1534/genetics.104.036830 (2005).

164 de Visser, J. A. & Krug, J. Empirical fitness landscapes and the predictability of evolution. *Nat Rev Genet* **15**, 480-490, doi:10.1038/nrg3744 (2014).

165 Maddamsetti, R., Lenski, R. E. & Barrick, J. E. Adaptation, Clonal Interference, and Frequency-Dependent Interactions in a Long-Term Evolution Experiment with Escherichia coli. *Genetics* **200**, 619-631, doi:10.1534/genetics.115.176677 (2015).

166 Gifford, D. R., Toll-Riera, M. & MacLean, R. C. Epistatic interactions between ancestral genotype and beneficial mutations shape evolvability in Pseudomonas aeruginosa. *Evolution* **70**, 1659-1666, doi:10.1111/evo.12958 (2016).

167 Mao, E. F., Lane, L., Lee, J. & Miller, J. H. Proliferation of mutators in A cell population. *J Bacteriol* **179**, 417-422, doi:10.1128/jb.179.2.417-422.1997 (1997).

168 Wistrand-Yuen, E. *et al.* Evolution of high-level resistance during low-level antibiotic exposure. *Nat Commun* **9**, 1599, doi:10.1038/s41467-018-04059-1 (2018).

169 Jahn, L. J., Munck, C., Ellabaan, M. M. H. & Sommer, M. O. A. Adaptive Laboratory Evolution of Antibiotic Resistance Using Different Selection Regimes Lead to Similar Phenotypes and Genotypes. *Front Microbiol* **8**, 816, doi:10.3389/fmicb.2017.00816 (2017).

170 Zhao, X. & Drlica, K. Restricting the selection of antibiotic-resistant mutant bacteria: measurement and potential use of the mutant selection window. *J Infect Dis* **185**, 561-565, doi:10.1086/338571 (2002).

171 Baquero, F. Resistance to quinolones in gram-negative microorganisms: mechanisms and prevention. *Eur Urol* **17 Suppl 1**, 3-12, doi:10.1159/000464084 (1990).

172 Baquero, F. & Negri, M. C. Selective compartments for resistant microorganisms in antibiotic gradients. *Bioessays* **19**, 731-736, doi:10.1002/bies.950190814 (1997).

173 Gullberg, E. *et al.* Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog* **7**, e1002158, doi:10.1371/journal.ppat.1002158 (2011).

174 Blazquez, J., Rodriguez-Beltran, J. & Matic, I. Antibiotic-Induced Genetic Variation: How It Arises and How It Can Be Prevented. *Annu Rev Microbiol* **72**, 209-230, doi:10.1146/annurev-micro-090817-062139 (2018).

175 Andersson, D. I. & Hughes, D. Microbiological effects of sublethal levels of antibiotics. *Nat Rev Microbiol* **12**, 465-478, doi:10.1038/nrmicro3270 (2014).

176 Martinez, J. L. Antibiotics and antibiotic resistance genes in natural environments. *Science* **321**, 365-367, doi:10.1126/science.1159483 (2008).

177 Danner, M. C., Robertson, A., Behrends, V. & Reiss, J. Antibiotic pollution in surface fresh waters: Occurrence and effects. *Sci Total Environ* **664**, 793-804, doi:10.1016/j.scitotenv.2019.01.406 (2019).

178 Wu, Y. L., Scott, E. M., Po, A. L. & Tariq, V. N. Development of resistance and cross-resistance in Pseudomonas aeruginosa exposed to subinhibitory antibiotic concentrations. *APMIS* **107**, 585-592 (1999).

179 Wong, A. Epistasis and the Evolution of Antimicrobial Resistance. *Front Microbiol* **8**, 246, doi:10.3389/fmicb.2017.00246 (2017).

180 Vogwill, T., Kojadinovic, M. & MacLean, R. C. Epistasis between antibiotic resistance mutations and genetic background shape the fitness effect of resistance across species of Pseudomonas. *Proc Biol Sci* **283**, doi:10.1098/rspb.2016.0151 (2016).

181 Blount, Z. D., Borland, C. Z. & Lenski, R. E. Historical contingency and the evolution of a key innovation in an experimental population of Escherichia coli. *Proc Natl Acad Sci U S A* **105**, 7899-7906, doi:10.1073/pnas.0803151105 (2008).

182 Novais, A. *et al.* Evolutionary trajectories of beta-lactamase CTX-M-1 cluster enzymes: predicting antibiotic resistance. *PLoS Pathog* **6**, e1000735, doi:10.1371/journal.ppat.1000735 (2010).

183 Salverda, M. L. *et al.* Initial mutations direct alternative pathways of protein evolution. *PLoS Genet* **7**, e1001321, doi:10.1371/journal.pgen.1001321 (2011).

184 Kryazhimskiy, S., Rice, D. P., Jerison, E. R. & Desai, M. M. Microbial evolution. Global epistasis makes adaptation predictable despite sequence-level stochasticity. *Science* **344**, 1519-1522, doi:10.1126/science.1250939 (2014).

185 Ward, H., Perron, G. G. & Maclean, R. C. The cost of multiple drug resistance in Pseudomonas aeruginosa. *J Evol Biol* **22**, 997-1003, doi:10.1111/j.1420-9101.2009.01712.x (2009).

186 Hoeksema, M., Jonker, M. J., Brul, S. & Ter Kuile, B. H. Effects of a previously selected antibiotic resistance on mutations acquired during development of a second resistance in Escherichia coli. *BMC Genomics* **20**, 284, doi:10.1186/s12864-019-5648-7 (2019).

187 Martinez-Solano, L., Macia, M. D., Fajardo, A., Oliver, A. & Martinez, J. L. Chronic Pseudomonas aeruginosa infection in chronic obstructive pulmonary disease. *Clin Infect Dis* **47**, 1526-1533, doi:10.1086/593186 (2008).

188 Silby, M. W., Winstanley, C., Godfrey, S. A., Levy, S. B. & Jackson, R. W. Pseudomonas genomes: diverse and adaptable. *FEMS Microbiol Rev* **35**, 652-680, doi:10.1111/j.1574-6976.2011.00269.x (2011).

189 Green, S. K., Schroth, M. N., Cho, J. J., Kominos, S. K. & Vitanza-jack, V. B. Agricultural plants and soil as a reservoir for Pseudomonas aeruginosa. *Appl Microbiol* **28**, 987-991 (1974).

190 Youenou, B., Brothier, E. & Nazaret, S. Diversity among strains of Pseudomonas aeruginosa from manure and soil, evaluated by multiple locus variable number tandem repeat analysis and antibiotic resistance profiles. *Res Microbiol* **165**, 2-13, doi:10.1016/j.resmic.2013.10.004 (2014).

191 Howard, I., Espigares, E., Lardelli, P., Martin, J. L. & Espigares, M. Evaluation of microbiological and physicochemical indicators for wastewater treatment. *Environ Toxicol* **19**, 241-249, doi:10.1002/tox.20016 (2004).

192 Myers, G. E. Survival of pathogenic bacteria in crude oil. *J Environ Pathol Toxicol Oncol* **5**, 79-87 (1984).

193 Crone, S. *et al.* The environmental occurrence of Pseudomonas aeruginosa. *APMIS*, doi:10.1111/apm.13010 (2019).

194 Weir, T. L. *et al.* Global gene expression profiles suggest an important role for nutrient acquisition in early pathogenesis in a plant model of Pseudomonas aeruginosa infection. *Appl Environ Microbiol* **74**, 5784-5791, doi:10.1128/AEM.00860-08 (2008).

195 Walker, T. S. *et al.* Pseudomonas aeruginosa-plant root interactions. Pathogenicity, biofilm formation, and root exudation. *Plant Physiol* **134**, 320-331, doi:10.1104/pp.103.027888 (2004).

196 Ruiz-Diez, B., Sanchez, P., Baquero, F., Martinez, J. L. & Navas, A. Differential interactions within the Caenorhabditis elegans-Pseudomonas aeruginosa pathogenesis model. *J Theor Biol* **225**, 469-476, doi:10.1016/s0022-5193(03)00288-1 (2003).

197 Clatworthy, A. E. *et al.* Pseudomonas aeruginosa infection of zebrafish involves both host and pathogen determinants. *Infect Immun* **77**, 1293-1303, doi:10.1128/IAI.01181-08 (2009).

198 Obritsch, M. D., Fish, D. N., MacLaren, R. & Jung, R. Nosocomial infections due to multidrug-resistant Pseudomonas aeruginosa: epidemiology and treatment options. *Pharmacotherapy* **25**, 1353-1364, doi:10.1592/phco.2005.25.10.1353 (2005).

199 Talwalkar, J. S. & Murray, T. S. The Approach to Pseudomonas aeruginosa in Cystic Fibrosis. *Clin Chest Med* **37**, 69-81, doi:10.1016/j.ccm.2015.10.004 (2016).

200 Samonis, G. *et al.* Characteristics, risk factors and outcomes of adult cancer patients with extensively drug-resistant Pseudomonas aeruginosa infections. *Infection* **42**, 721-728, doi:10.1007/s15010-014-0635-z (2014).

201 Kc, R. *et al.* Opportunistic Respiratory Infections in HIV Patients Attending Sukraraj Tropical and Infectious Diseases Hospital in Kathmandu, Nepal. *HIV AIDS (Auckl)* **11**, 357-367, doi:10.2147/HIV.S229531 (2019).

202 Turner, K. H., Everett, J., Trivedi, U., Rumbaugh, K. P. & Whiteley, M. Requirements for Pseudomonas aeruginosa acute burn and chronic surgical wound infection. *PLoS Genet* **10**, e1004518, doi:10.1371/journal.pgen.1004518 (2014).

203 Kaye, K. S. & Pogue, J. M. Infections Caused by Resistant Gram-Negative Bacteria: Epidemiology and Management. *Pharmacotherapy* **35**, 949-962, doi:10.1002/phar.1636 (2015).

204 Cole, S. J., Records, A. R., Orr, M. W., Linden, S. B. & Lee, V. T. Catheter-associated urinary tract infection by Pseudomonas aeruginosa is mediated by exopolysaccharide-independent biofilms. *Infect Immun* **82**, 2048-2058, doi:10.1128/IAI.01652-14 (2014).

205 Gbejuade, H. O., Lovering, A. M. & Webb, J. C. The role of microbial biofilms in prosthetic joint infections. *Acta Orthop* **86**, 147-158, doi:10.3109/17453674.2014.966290 (2015).

206 Hoiby, N., Ciofu, O. & Bjarnsholt, T. Pseudomonas aeruginosa biofilms in cystic fibrosis. *Future Microbiol* **5**, 1663-1674, doi:10.2217/fmb.10.125 (2010).

207 Poole, K. Multidrug efflux pumps and antimicrobial resistance in Pseudomonas aeruginosa and related organisms. *J Mol Microbiol Biotechnol* **3**, 255-264 (2001).

208 Pankey, G. A. Tigecycline. *J Antimicrob Chemother* **56**, 470-480, doi:10.1093/jac/dki248 (2005).

209 Burkhardt, O. *et al.* Once-daily tobramycin in cystic fibrosis: better for clinical outcome than thrice-daily tobramycin but more resistance development? *J Antimicrob Chemother* **58**, 822-829, doi:10.1093/jac/dkl328 (2006).

210 Merlo, C. A. *et al.* Incidence and risk factors for multiple antibiotic-resistant Pseudomonas aeruginosa in cystic fibrosis. *Chest* **132**, 562-568, doi:10.1378/chest.06-2888 (2007).

211 Holbrook, S. Y. L. & Garneau-Tsodikova, S. Evaluation of Aminoglycoside and Carbapenem Resistance in a Collection of Drug-Resistant Pseudomonas aeruginosa Clinical Isolates. *Microb Drug Resist* **24**, 1020-1030, doi:10.1089/mdr.2017.0101 (2018).

212 Bellido, F., Martin, N. L., Siehnel, R. J. & Hancock, R. E. Reevaluation, using intact cells, of the exclusion limit and role of porin OprF in Pseudomonas aeruginosa outer membrane permeability. *J Bacteriol* **174**, 5196-5203, doi:10.1128/jb.174.16.5196-5203.1992 (1992).

213 Hancock, R. E. & Brinkman, F. S. Function of pseudomonas porins in uptake and efflux. *Annu Rev Microbiol* **56**, 17-38, doi:10.1146/annurev.micro.56.012302.160310 (2002).

214 Pang, Z., Raudonis, R., Glick, B. R., Lin, T. J. & Cheng, Z. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies. *Biotechnol Adv* **37**, 177-192, doi:10.1016/j.biotechadv.2018.11.013 (2019).

215 Poole, K. Aminoglycoside resistance in Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **49**, 479-487, doi:10.1128/AAC.49.2.479-487.2005 (2005).

216 Wolter, D. J. & Lister, P. D. Mechanisms of beta-lactam resistance among Pseudomonas aeruginosa. *Curr Pharm Des* **19**, 209-222 (2013).

217 Stover, C. K. *et al.* Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature* **406**, 959-964, doi:10.1038/35023079 (2000).

218 Poole, K. *et al.* Overexpression of the mexC-mexD-oprJ efflux operon in nfxB-type multidrug-resistant strains of Pseudomonas aeruginosa. *Mol Microbiol* **21**, 713-724, doi:10.1046/j.1365-2958.1996.281397.x (1996).

219 Kohler, T. *et al.* Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of Pseudomonas aeruginosa. *Mol Microbiol* **23**, 345-354, doi:10.1046/j.1365-2958.1997.2281594.x (1997).

220 Masuda, N. *et al.* Contribution of the MexX-MexY-oprM efflux system to intrinsic resistance in Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **44**, 2242-2246, doi:10.1128/aac.44.9.2242-2246.2000 (2000).

221 Ramirez, M. S. & Tolmasky, M. E. Aminoglycoside modifying enzymes. *Drug Resist Updat* **13**, 151-171, doi:10.1016/j.drup.2010.08.003 (2010).
222 Cao, L., Srikumar, R. & Poole, K. MexAB-OprM hyperexpression in NalC-type multidrug-resistant Pseudomonas aeruginosa: identification and characterization of the nalC gene encoding a repressor of PA3720-PA3719. *Mol Microbiol* **53**, 1423-1436, doi:10.1111/j.1365-2958.2004.04210.x (2004).
223 Morita, Y., Cao, L., Gould, V. C., Avison, M. B. & Poole, K. nalD encodes a second repressor of the mexAB-oprM multidrug efflux operon of Pseudomonas aeruginosa. *J Bacteriol* **188**, 8649-8654, doi:10.1128/JB.01342-06 (2006).
224 Sobel, M. L., Hocquet, D., Cao, L., Plesiat, P. & Poole, K. Mutations in PA3574 (nalD) lead to increased MexAB-OprM expression and multidrug resistance in laboratory and clinical isolates of Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **49**, 1782-1786, doi:10.1128/AAC.49.5.1782-1786.2005 (2005).
225 Llanes, C. *et al.* Clinical strains of Pseudomonas aeruginosa overproducing MexAB-OprM and MexXY efflux pumps simultaneously. *Antimicrob Agents Chemother* **48**, 1797-1802, doi:10.1128/aac.48.5.1797-1802.2004 (2004).
226 Jeannot, K. *et al.* Resistance and virulence of Pseudomonas aeruginosa clinical strains overproducing the MexCD-OprJ efflux pump. *Antimicrob Agents Chemother* **52**, 2455-2462, doi:10.1128/AAC.01107-07 (2008).
227 Balcewich, M. D. *et al.* Crystal structure of the AmpR effector binding domain provides insight into the molecular regulation of inducible ampc beta-lactamase. *J Mol Biol* **400**, 998-1010, doi:10.1016/j.jmb.2010.05.040 (2010).
228 Alvarez-Ortega, C., Wiegand, I., Olivares, J., Hancock, R. E. & Martinez, J. L. Genetic determinants involved in the susceptibility of Pseudomonas aeruginosa to beta-lactam antibiotics. *Antimicrob Agents Chemother* **54**, 4159-4167, doi:10.1128/AAC.00257-10 (2010).
229 Aguilera Rossi, C. G., Gomez-Puertas, P. & Ayala Serrano, J. A. In vivo functional and molecular characterization of the Penicillin-Binding Protein 4 (DacB) of Pseudomonas aeruginosa. *BMC Microbiol* **16**, 234, doi:10.1186/s12866-016-0853-x (2016).
230 Bruchmann, S., Dotsch, A., Nouri, B., Chaberny, I. F. & Haussler, S. Quantitative contributions of target alteration and decreased drug accumulation to Pseudomonas aeruginosa fluoroquinolone resistance. *Antimicrob Agents Chemother* **57**, 1361-1368, doi:10.1128/AAC.01581-12 (2013).
231 Cabot, G., Florit-Mendoza, L., Sanchez-Diener, I., Zamorano, L. & Oliver, A. Deciphering beta-lactamase-independent beta-lactam resistance evolution trajectories in Pseudomonas aeruginosa. *J Antimicrob Chemother* **73**, 3322-3331, doi:10.1093/jac/dky364 (2018).
232 Hong, D. J. *et al.* Epidemiology and Characteristics of Metallo-beta-Lactamase-Producing Pseudomonas aeruginosa. *Infect Chemother* **47**, 81-97, doi:10.3947/ic.2015.47.2.81 (2015).
233 Poirel, L. *et al.* Characterization of Class 1 integrons from Pseudomonas aeruginosa that contain the bla(VIM-2) carbapenem-hydrolyzing beta-lactamase gene and of two novel aminoglycoside resistance gene cassettes. *Antimicrob Agents Chemother* **45**, 546-552, doi:10.1128/AAC.45.2.546-552.2001 (2001).
234 Taylor, P. K., Yeung, A. T. & Hancock, R. E. Antibiotic resistance in Pseudomonas aeruginosa biofilms: towards the development of novel anti-biofilm therapies. *J Biotechnol* **191**, 121-130, doi:10.1016/j.jbiotec.2014.09.003 (2014).
235 Smith, R. S. & Iglewski, B. H. P. aeruginosa quorum-sensing systems and virulence. *Curr Opin Microbiol* **6**, 56-60, doi:10.1016/s1369-5274(03)00008-0 (2003).
236 Overhage, J., Bains, M., Brazas, M. D. & Hancock, R. E. Swarming of Pseudomonas aeruginosa is a complex adaptation leading to increased production of virulence

factors and antibiotic resistance. *J Bacteriol* **190**, 2671-2679, doi:10.1128/JB.01659-07 (2008).

237 Swift, S. *et al.* Quorum sensing as a population-density-dependent determinant of bacterial physiology. *Adv Microb Physiol* **45**, 199-270, doi:10.1016/s0065-2911(01)45005-3 (2001).

238 Williams, P. & Camara, M. Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules. *Curr Opin Microbiol* **12**, 182-191, doi:10.1016/j.mib.2009.01.005 (2009).

239 Wagner, V. E., Gillis, R. J. & Iglewski, B. H. Transcriptome analysis of quorum-sensing regulation and virulence factor expression in Pseudomonas aeruginosa. *Vaccine* **22 Suppl 1**, S15-20, doi:10.1016/j.vaccine.2004.08.011 (2004).

240 Dietrich, L. E., Price-Whelan, A., Petersen, A., Whiteley, M. & Newman, D. K. The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of Pseudomonas aeruginosa. *Mol Microbiol* **61**, 1308-1321, doi:10.1111/j.1365-2958.2006.05306.x (2006).

241 Mayer-Hamblett, N. *et al.* Pseudomonas aeruginosa in vitro phenotypes distinguish cystic fibrosis infection stages and outcomes. *Am J Respir Crit Care Med* **190**, 289-297, doi:10.1164/rccm.201404-0681OC (2014).

242 Rodriguez-Rojas, A. *et al.* Inactivation of the hmgA gene of Pseudomonas aeruginosa leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection. *Microbiology* **155**, 1050-1057, doi:10.1099/mic.0.024745-0 (2009).

243 Cheer, S. M., Waugh, J. & Noble, S. Inhaled tobramycin (TOBI): a review of its use in the management of Pseudomonas aeruginosa infections in patients with cystic fibrosis. *Drugs* **63**, 2501-2520, doi:10.2165/00003495-200363220-00015 (2003).

244 Migiyama, Y. *et al.* Aerosolized tobramycin for Pseudomonas aeruginosa ventilator-associated pneumonia in patients with acute respiratory distress syndrome. *Pulm Pharmacol Ther* **45**, 142-147, doi:10.1016/j.pupt.2017.04.008 (2017).

245 Bassetti, M., Vena, A., Croxatto, A., Righi, E. & Guery, B. How to manage Pseudomonas aeruginosa infections. *Drugs Context* **7**, 212527, doi:10.7573/dic.212527 (2018).

246 Gaspar, M. C., Couet, W., Olivier, J. C., Pais, A. A. & Sousa, J. J. Pseudomonas aeruginosa infection in cystic fibrosis lung disease and new perspectives of treatment: a review. *Eur J Clin Microbiol Infect Dis* **32**, 1231-1252, doi:10.1007/s10096-013-1876-y (2013).

247 Wagner, S. *et al.* Novel Strategies for the Treatment of Pseudomonas aeruginosa Infections. *J Med Chem* **59**, 5929-5969, doi:10.1021/acs.jmedchem.5b01698 (2016).

248 Hancock, R. E. & Speert, D. P. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment. *Drug Resist Updat* **3**, 247-255, doi:10.1054/drup.2000.0152 (2000).

249 Escola-Verge, L., Pigrau, C. & Almirante, B. Ceftolozane/tazobactam for the treatment of complicated intra-abdominal and urinary tract infections: current perspectives and place in therapy. *Infect Drug Resist* **12**, 1853-1867, doi:10.2147/IDR.S180905 (2019).

250 Liscio, J. L., Mahoney, M. V. & Hirsch, E. B. Ceftolozane/tazobactam and ceftazidime/avibactam: two novel beta-lactam/beta-lactamase inhibitor combination agents for the treatment of resistant Gram-negative bacterial infections. *Int J Antimicrob Agents* **46**, 266-271, doi:10.1016/j.ijantimicag.2015.05.003 (2015).

251 Aktas, Z., Kayacan, C. & Oncul, O. In vitro activity of avibactam (NXL104) in combination with beta-lactams against Gram-negative bacteria, including OXA-48 beta-lactamase-producing Klebsiella pneumoniae. *Int J Antimicrob Agents* **39**, 86-89, doi:10.1016/j.ijantimicag.2011.09.012 (2012).

252 Hidalgo, J. A., Vinluan, C. M. & Antony, N. Ceftazidime/avibactam: a novel cephalosporin/nonbeta-lactam beta-lactamase inhibitor for the treatment of

complicated urinary tract infections and complicated intra-abdominal infections. *Drug Des Devel Ther* **10**, 2379-2386, doi:10.2147/DDDT.S110946 (2016).

253 Fraile-Ribot, P. A. *et al.* Activity of Imipenem-Relebactam against a Large Collection of Pseudomonas aeruginosa Clinical Isolates and Isogenic beta-Lactam-Resistant Mutants. *Antimicrob Agents Chemother* **64**, doi:10.1128/AAC.02165-19 (2020).

254 Khil, P. P. *et al.* Dynamic Emergence of Mismatch Repair Deficiency Facilitates Rapid Evolution of Ceftazidime-Avibactam Resistance in Pseudomonas aeruginosa Acute Infection. *mBio* **10**, doi:10.1128/mBio.01822-19 (2019).

255 Garcia-Cabrera, E. *et al.* Superinfection during treatment of nosocomial infections with tigecycline. *Eur J Clin Microbiol Infect Dis* **29**, 867-871, doi:10.1007/s10096-010-0942-y (2010).

256 Ulu-Kilic, A. *et al.* Increasing frequency of Pseudomonas aeruginosa infections during tigecycline use. *J Infect Dev Ctries* **9**, 309-312, doi:10.3855/jidc.4700 (2015).

257 Katsiari, M. *et al.* Characteristics of superinfections during treatment with tigecycline. *J Chemother* **28**, 110-115, doi:10.1080/1120009X.2015.1118184 (2016).

258 Martinez, J. L., Coque, T. M. & Baquero, F. What is a resistance gene? Ranking risk in resistomes. *Nat Rev Microbiol* **13**, 116-123, doi:10.1038/nrmicro3399 (2015).

259 Dean, C. R., Visalli, M. A., Projan, S. J., Sum, P. E. & Bradford, P. A. Efflux-mediated resistance to tigecycline (GAR-936) in Pseudomonas aeruginosa PAO1. *Antimicrob Agents Chemother* **47**, 972-978, doi:10.1128/aac.47.3.972-978.2003 (2003).

260 Williams, P. Quorum sensing: an emerging target for antibacterial chemotherapy? *Expert Opin Ther Targets* **6**, 257-274, doi:10.1517/14728222.6.3.257 (2002).

261 Brannon, J. R. & Hadjifrangiskou, M. The arsenal of pathogens and antivirulence therapeutic strategies for disarming them. *Drug Des Devel Ther* **10**, 1795-1806, doi:10.2147/DDDT.S98939 (2016).

262 Galdino, A. C. M. *et al.* Anti-Virulence Strategy against the Multidrug-Resistant Bacterial Pathogen Pseudomonas aeruginosa: Pseudolysin (Elastase B) as a Potential Druggable Target. *Curr Protein Pept Sci* **20**, 471-487, doi:10.2174/1389203720666190207100415 (2019).

263 Imperi, F., Fiscarelli, E. V., Visaggio, D., Leoni, L. & Visca, P. Activity and Impact on Resistance Development of Two Antivirulence Fluoropyrimidine Drugs in Pseudomonas aeruginosa. *Front Cell Infect Microbiol* **9**, 49, doi:10.3389/fcimb.2019.00049 (2019).

264 Martinez, J. L. & Baquero, F. Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance. *Clin Microbiol Rev* **15**, 647-679, doi:10.1128/cmr.15.4.647-679.2002 (2002).

265 Liberati, N. T. *et al.* An ordered, nonredundant library of Pseudomonas aeruginosa strain PA14 transposon insertion mutants. *Proc Natl Acad Sci U S A* **103**, 2833-2838, doi:10.1073/pnas.0511100103 (2006).

266 Castanheira, M., Doyle, T. B., Smith, C. J., Mendes, R. E. & Sader, H. S. Combination of MexAB-OprM overexpression and mutations in efflux regulators, PBPs and chaperone proteins is responsible for ceftazidime/avibactam resistance in Pseudomonas aeruginosa clinical isolates from US hospitals. *J Antimicrob Chemother* **74**, 2588-2595, doi:10.1093/jac/dkz243 (2019).

267 Nunn, D., Bergman, S. & Lory, S. Products of three accessory genes, pilB, pilC, and pilD, are required for biogenesis of Pseudomonas aeruginosa pili. *J Bacteriol* **172**, 2911-2919, doi:10.1128/jb.172.6.2911-2919.1990 (1990).

268 Wargo, M. J., Szwergold, B. S. & Hogan, D. A. Identification of two gene clusters and a transcriptional regulator required for Pseudomonas aeruginosa glycine betaine catabolism. *J Bacteriol* **190**, 2690-2699, doi:10.1128/JB.01393-07 (2008).

269 Contreras, A. *et al.* The product of the nitrogen fixation regulatory gene nfrX of Azotobacter vinelandii is functionally and structurally homologous to the

uridylyltransferase encoded by glnD in enteric bacteria. *J Bacteriol* **173**, 7741-7749, doi:10.1128/jb.173.24.7741-7749.1991 (1991).

270 Huang, H. *et al.* An integrated genomic regulatory network of virulence-related transcriptional factors in Pseudomonas aeruginosa. *Nat Commun* **10**, 2931, doi:10.1038/s41467-019-10778-w (2019).

271 Diez-Aguilar, M. *et al.* Use of Calgary and Microfluidic BioFlux Systems To Test the Activity of Fosfomycin and Tobramycin Alone and in Combination against Cystic Fibrosis Pseudomonas aeruginosa Biofilms. *Antimicrob Agents Chemother* **62**, doi:10.1128/AAC.01650-17 (2018).

272 McCaughey, G. *et al.* Fosfomycin and tobramycin in combination downregulate nitrate reductase genes narG and narH, resulting in increased activity against Pseudomonas aeruginosa under anaerobic conditions. *Antimicrob Agents Chemother* **57**, 5406-5414, doi:10.1128/AAC.00750-13 (2013).

273 Jansen, G., Barbosa, C. & Schulenburg, H. Experimental evolution as an efficient tool to dissect adaptive paths to antibiotic resistance. *Drug Resist Updat* **16**, 96-107, doi:10.1016/j.drup.2014.02.002 (2013).

274 Cabot, G. *et al.* Pseudomonas aeruginosa ceftolozane-tazobactam resistance development requires multiple mutations leading to overexpression and structural modification of AmpC. *Antimicrob Agents Chemother* **58**, 3091-3099, doi:10.1128/AAC.02462-13 (2014).

275 Baym, M. *et al.* Spatiotemporal microbial evolution on antibiotic landscapes. *Science* **353**, 1147-1151, doi:10.1126/science.aag0822 (2016).

276 Wong, A., Rodrigue, N. & Kassen, R. Genomics of adaptation during experimental evolution of the opportunistic pathogen Pseudomonas aeruginosa. *PLoS Genet* **8**, e1002928, doi:10.1371/journal.pgen.1002928 (2012).

277 Jorth, P. *et al.* Evolved Aztreonam Resistance Is Multifactorial and Can Produce Hypervirulence in Pseudomonas aeruginosa. *mBio* **8**, doi:10.1128/mBio.00517-17 (2017).

278 Scribner, M. R., Santos-Lopez, A., Marshall, C. W., Deitrick, C. & Cooper, V. S. Parallel Evolution of Tobramycin Resistance across Species and Environments. *mBio* **11**, doi:10.1128/mBio.00932-20 (2020).

279 Moxon, R., Bayliss, C. & Hood, D. Bacterial contingency loci: the role of simple sequence DNA repeats in bacterial adaptation. *Annu Rev Genet* **40**, 307-333, doi:10.1146/annurev.genet.40.110405.090442 (2006).

280 Arora, S. K., Wolfgang, M. C., Lory, S. & Ramphal, R. Sequence polymorphism in the glycosylation island and flagellins of Pseudomonas aeruginosa. *J Bacteriol* **186**, 2115-2122, doi:10.1128/jb.186.7.2115-2122.2004 (2004).

281 Muller, C., Plesiat, P. & Jeannot, K. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and beta-lactams in Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **55**, 1211-1221, doi:10.1128/AAC.01252-10 (2011).

282 Lopez-Causape, C., Cabot, G., Del Barrio-Tofino, E. & Oliver, A. The Versatile Mutational Resistome of Pseudomonas aeruginosa. *Front Microbiol* **9**, 685, doi:10.3389/fmicb.2018.00685 (2018).

283 Bricio-Moreno, L. *et al.* Evolutionary trade-offs associated with loss of PmrB function in host-adapted Pseudomonas aeruginosa. *Nat Commun* **9**, 2635, doi:10.1038/s41467-018-04996-x (2018).

284 Colque, C. A. *et al.* Hypermutator Pseudomonas aeruginosa Exploits Multiple Genetic Pathways To Develop Multidrug Resistance during Long-Term Infections in the Airways of Cystic Fibrosis Patients. *Antimicrob Agents Chemother* **64**, doi:10.1128/AAC.02142-19 (2020).

285 Bolard, A., Plesiat, P. & Jeannot, K. Mutations in Gene fusA1 as a Novel Mechanism of Aminoglycoside Resistance in Clinical Strains of Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **62**, doi:10.1128/AAC.01835-17 (2018).

286 Yen, P. & Papin, J. A. History of antibiotic adaptation influences microbial evolutionary dynamics during subsequent treatment. *PLoS Biol* **15**, e2001586, doi:10.1371/journal.pbio.2001586 (2017).

287 Purssell, A. & Poole, K. Functional characterization of the NfxB repressor of the mexCD-oprJ multidrug efflux operon of Pseudomonas aeruginosa. *Microbiology* **159**, 2058-2073, doi:10.1099/mic.0.069286-0 (2013).

288 Masuda, N. *et al.* Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **44**, 3322-3327, doi:10.1128/aac.44.12.3322-3327.2000 (2000).

289 Elkins, C. A. & Nikaido, H. Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of Escherichia coli is determined predominantly by two large periplasmic loops. *J Bacteriol* **184**, 6490-6498, doi:10.1128/jb.184.23.6490-6499.2002 (2002).

290 Kiratisin, P., Tucker, K. D. & Passador, L. LasR, a transcriptional activator of Pseudomonas aeruginosa virulence genes, functions as a multimer. *J Bacteriol* **184**, 4912-4919, doi:10.1128/jb.184.17.4912-4919.2002 (2002).

291 Porse, A., Jahn, L. J., Ellabaan, M. M. H. & Sommer, M. O. A. Dominant resistance and negative epistasis can limit the co-selection of de novo resistance mutations and antibiotic resistance genes. *Nat Commun* **11**, 1199, doi:10.1038/s41467-020-15080-8 (2020).

292 Wilder, C. N., Allada, G. & Schuster, M. Instantaneous within-patient diversity of Pseudomonas aeruginosa quorum-sensing populations from cystic fibrosis lung infections. *Infect Immun* **77**, 5631-5639, doi:10.1128/IAI.00755-09 (2009).

293 LaFayette, S. L. *et al.* Cystic fibrosis-adapted Pseudomonas aeruginosa quorum sensing lasR mutants cause hyperinflammatory responses. *Sci Adv* **1**, doi:10.1126/sciadv.1500199 (2015).

294 D'Argenio, D. A. *et al.* Growth phenotypes of Pseudomonas aeruginosa lasR mutants adapted to the airways of cystic fibrosis patients. *Mol Microbiol* **64**, 512-533, doi:10.1111/j.1365-2958.2007.05678.x (2007).

295 Heurlier, K. *et al.* Quorum-sensing-negative (lasR) mutants of Pseudomonas aeruginosa avoid cell lysis and death. *J Bacteriol* **187**, 4875-4883, doi:10.1128/JB.187.14.4875-4883.2005 (2005).

296 Ruparell, A. *et al.* The fitness burden imposed by synthesising quorum sensing signals. *Sci Rep* **6**, 33101, doi:10.1038/srep33101 (2016).

297 Gnanasekaran, G., Lim, J. Y. & Hwang, I. Disappearance of Quorum Sensing in Burkholderia glumae During Experimental Evolution. *Microb Ecol*, doi:10.1007/s00248-019-01445-0 (2019).

298 Azimi, S. *et al.* Allelic polymorphism shapes community function in evolving Pseudomonas aeruginosa populations. *ISME J*, doi:10.1038/s41396-020-0652-0 (2020).

299 Pelchovich, G., Zhuravlev, A. & Gophna, U. Effect of ribosome-targeting antibiotics on streptomycin-resistant Mycobacterium mutants in the rpsL gene. *Int J Antimicrob Agents* **42**, 129-132, doi:10.1016/j.ijantimicag.2013.04.001 (2013).

300 Zhao, Q. & Poole, K. Differential effects of mutations in tonB1 on intrinsic multidrug resistance and iron acquisition in Pseudomonas aeruginosa. *J Bacteriol* **184**, 2045-2049, doi:10.1128/jb.184.7.2045-2049.2002 (2002).

301 Matsuo, Y., Eda, S., Gotoh, N., Yoshihara, E. & Nakae, T. MexZ-mediated regulation of mexXY multidrug efflux pump expression in Pseudomonas aeruginosa by binding on the mexZ-mexX intergenic DNA. *FEMS Microbiol Lett* **238**, 23-28, doi:10.1016/j.femsle.2004.07.010 (2004).

302 Oliver, A., Baquero, F. & Blazquez, J. The mismatch repair system (mutS, mutL and uvrD genes) in Pseudomonas aeruginosa: molecular characterization of naturally occurring mutants. *Mol Microbiol* **43**, 1641-1650, doi:10.1046/j.1365-2958.2002.02855.x (2002).

303 Long, H., Miller, S. F., Williams, E. & Lynch, M. Specificity of the DNA Mismatch Repair System (MMR) and Mutagenesis Bias in Bacteria. *Mol Biol Evol* **35**, 2414-2421, doi:10.1093/molbev/msy134 (2018).

304 Macfarlane, E. L. A., Kwasnicka, A. & Hancock, R. E. W. Role of Pseudomonas aeruginosa PhoP-phoQ in resistance to antimicrobial cationic peptides and aminoglycosides. *Microbiology* **146 (Pt 10)**, 2543-2554, doi:10.1099/00221287-146-10-2543 (2000).

305 Ben Jeddou, F. *et al.* Adaptive and mutational responses to peptide dendrimer antimicrobials in Pseudomonas aeruginosa. *Antimicrob Agents Chemother*, doi:10.1128/AAC.02040-19 (2020).

306 Chen, L. & Duan, K. A PhoPQ-Regulated ABC Transporter System Exports Tetracycline in Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **60**, 3016-3024, doi:10.1128/AAC.02986-15 (2016).

307 Webber, M. A. *et al.* Parallel evolutionary pathways to antibiotic resistance selected by biocide exposure. *J Antimicrob Chemother* **70**, 2241-2248, doi:10.1093/jac/dkv109 (2015).

308 Li, Q. J. *et al.* Compensatory Mutations of Rifampin Resistance Are Associated with Transmission of Multidrug-Resistant Mycobacterium tuberculosis Beijing Genotype Strains in China. *Antimicrob Agents Chemother* **60**, 2807-2812, doi:10.1128/AAC.02358-15 (2016).

309 Hughes, D. & Andersson, D. I. Selection of resistance at lethal and non-lethal antibiotic concentrations. *Curr Opin Microbiol* **15**, 555-560, doi:10.1016/j.mib.2012.07.005 (2012).

310 Smith, W. D. *et al.* Current and future therapies for Pseudomonas aeruginosa infection in patients with cystic fibrosis. *FEMS Microbiol Lett* **364**, doi:10.1093/femsle/fnx121 (2017).

311 Hoffmann, M. *et al.* Metabolism, excretion, and pharmacokinetics of [14C]tigecycline, a first-in-class glycylcycline antibiotic, after intravenous infusion to healthy male subjects. *Drug Metab Dispos* **35**, 1543-1553, doi:10.1124/dmd.107.015735 (2007).

312 Tian, Z., Liu, R., Zhang, H., Yang, M. & Zhang, Y. Developmental dynamics of antibiotic resistome in aerobic biofilm microbiota treating wastewater under stepwise increasing tigecycline concentrations. *Environ Int* **131**, 105008, doi:10.1016/j.envint.2019.105008 (2019).

313 Rundell, E. A., Commodore, N., Goodman, A. L. & Kazmierczak, B. I. A screen for antibiotic resistance determinants reveals a fitness cost of the flagellum in Pseudomonas aeruginosa. *J Bacteriol*, doi:10.1128/JB.00682-19 (2019).

314 Ahmed, M. N., Porse, A., Sommer, M. O. A., Hoiby, N. & Ciofu, O. Evolution of Antibiotic Resistance in Biofilm and Planktonic Pseudomonas aeruginosa Populations Exposed to Subinhibitory Levels of Ciprofloxacin. *Antimicrob Agents Chemother* **62**, doi:10.1128/AAC.00320-18 (2018).

315 Martinez, J. L. & Baquero, F. Mutation frequencies and antibiotic resistance. *Antimicrob Agents Chemother* **44**, 1771-1777, doi:10.1128/aac.44.7.1771-1777.2000 (2000).

316 Canton, R., Gonzalez-Alba, J. M. & Galan, J. C. CTX-M Enzymes: Origin and Diffusion. *Front Microbiol* **3**, 110, doi:10.3389/fmicb.2012.00110 (2012).

317 Toussaint, K. A. & Gallagher, J. C. beta-lactam/beta-lactamase inhibitor combinations: from then to now. *Ann Pharmacother* **49**, 86-98, doi:10.1177/1060028014556652 (2015).

318 Lahiri, S. D. *et al.* Selection and molecular characterization of ceftazidime/avibactam-resistant mutants in Pseudomonas aeruginosa strains containing derepressed AmpC. *J Antimicrob Chemother* **70**, 1650-1658, doi:10.1093/jac/dkv004 (2015).

319 Hocquet, D. *et al.* Pyomelanin-producing Pseudomonas aeruginosa selected during chronic infections have a large chromosomal deletion which confers resistance to pyocins. *Environ Microbiol* **18**, 3482-3493, doi:10.1111/1462-2920.13336 (2016).

320 Olsson, A. *et al.* Evaluation of the efficacy of antibiotic combinations against multidrug-resistant Pseudomonas aeruginosa in automated time-lapse microscopy and static time-kill experiments. *Antimicrob Agents Chemother*, doi:10.1128/AAC.02111-19 (2020).

321 Mulet, X. *et al.* Antagonistic interactions of Pseudomonas aeruginosa antibiotic resistance mechanisms in planktonic but not biofilm growth. *Antimicrob Agents Chemother* **55**, 4560-4568, doi:10.1128/AAC.00519-11 (2011).

322 Gifford, D. R. *et al.* Identifying and exploiting genes that potentiate the evolution of antibiotic resistance. *Nat Ecol Evol* **2**, 1033-1039, doi:10.1038/s41559-018-0547-x (2018).

323 Chalhoub, H. *et al.* High-level resistance to meropenem in clinical isolates of Pseudomonas aeruginosa in the absence of carbapenemases: role of active efflux and porin alterations. *Int J Antimicrob Agents* **48**, 740-743, doi:10.1016/j.ijantimicag.2016.09.012 (2016).

324 Pan, Y. P., Xu, Y. H., Wang, Z. X., Fang, Y. P. & Shen, J. L. Overexpression of MexAB-OprM efflux pump in carbapenem-resistant Pseudomonas aeruginosa. *Arch Microbiol* **198**, 565-571, doi:10.1007/s00203-016-1215-7 (2016).

325 Castanheira, M., Mills, J. C., Farrell, D. J. & Jones, R. N. Mutation-driven beta-lactam resistance mechanisms among contemporary ceftazidime-nonsusceptible Pseudomonas aeruginosa isolates from U.S. hospitals. *Antimicrob Agents Chemother* **58**, 6844-6850, doi:10.1128/AAC.03681-14 (2014).

326 Kos, V. N. *et al.* The resistome of Pseudomonas aeruginosa in relationship to phenotypic susceptibility. *Antimicrob Agents Chemother* **59**, 427-436, doi:10.1128/AAC.03954-14 (2015).

327 Cabot, G. *et al.* Genetic markers of widespread extensively drug-resistant Pseudomonas aeruginosa high-risk clones. *Antimicrob Agents Chemother* **56**, 6349-6357, doi:10.1128/AAC.01388-12 (2012).

328 Baym, M., Stone, L. K. & Kishony, R. Multidrug evolutionary strategies to reverse antibiotic resistance. *Science* **351**, aad3292, doi:10.1126/science.aad3292 (2016).

329 Barbosa, C. *et al.* Alternative Evolutionary Paths to Bacterial Antibiotic Resistance Cause Distinct Collateral Effects. *Mol Biol Evol* **34**, 2229-2244, doi:10.1093/molbev/msx158 (2017).

330 Workentine, M. L. *et al.* Phenotypic heterogeneity of Pseudomonas aeruginosa populations in a cystic fibrosis patient. *PLoS One* **8**, e60225, doi:10.1371/journal.pone.0060225 (2013).

331 Papp-Wallace, K. M. *et al.* Ceftazidime-Avibactam in Combination With Fosfomycin: A Novel Therapeutic Strategy Against Multidrug-Resistant Pseudomonas aeruginosa. *J Infect Dis* **220**, 666-676, doi:10.1093/infdis/jiz149 (2019).

332 Kumon, H., Ono, N., Iida, M. & Nickel, J. C. Combination effect of fosfomycin and ofloxacin against Pseudomonas aeruginosa growing in a biofilm. *Antimicrob Agents Chemother* **39**, 1038-1044, doi:10.1128/aac.39.5.1038 (1995).

333 Drusano, G. L. *et al.* The Combination of Fosfomycin plus Meropenem Is Synergistic for Pseudomonas aeruginosa PAO1 in a Hollow-Fiber Infection Model. *Antimicrob Agents Chemother* **62**, doi:10.1128/AAC.01682-18 (2018).